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Brain Reserpine Levels Following Large and Small Doses of Reserpine-H^3. (23856)

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Previous studies on fate and distribution of reserpine have consistently demonstrated lack of correlation between pharmacological effects and tissue concentrations(1,2,3). Samples of radioactive reserpine used in several studies unfortunately had C^{14} introduced into the trimethoxybenzoic acid portion of the molecule. Since it was recognized that a somewhat different pattern might be obtained if the methyl reserpate portion of the drug were labeled, attention was drawn to the method of Rowland and Wolfgang(4) for randomly labeling organic compounds with tritium. Application of this method to the labeling of reserpine proved successful and the results of some studies utilizing this material in guinea pigs are recorded below. Parts of this work have appeared in abstract form(5,6).

Methods. Preparation of tritium-labeled reserpine: 9.9 g of reserpine were mixed with 0.30 g of lithium carbonate and irradiated with neutrons for 10 days in the reactor at Brookhaven National Laboratory. The material was extracted with benzene and chromatographed on alumina(1). A yield of 500 mg of tritiated reserpine (reserpine-H^3) was obtained and shown to be pure by paper chromatography(7).* It had a specific activity of 7.8×10^5 dpm/mg with an isotope distribution in the trimethoxybenzoic acid

* We wish to thank Dr. R. A. Lucas of Chemical Research Division, CIBA Pharmaceutical Products Inc., for purification of the tritiated reserpine.

and methyl reserpate portions of 35 and 65% respectively.

Exp. I: Twelve male guinea pigs weighing approximately 350 g were injected intravenously with 2 mg of tritiated reserpine and sacrificed in pairs after ⅓, 1, 2, 4, 8, and 24 hours. The blood was heparinized on collection and quickly separated into plasma and cells. Urine was collected when possible. The brains and livers were removed and stored in vials embedded in dry ice until analyzed. The tissues were treated as described previously (2) so as to yield fractions B and C. After the removal of fractions B and C the aqueous residue was brought to pH 8 with alkali and extracted with chloroform to yield fraction MR. Fraction B contained all of the unmetabolized reserpine, fraction C all of the trimethoxybenzoic acid (TMBA) and fraction MR all of the methyl reserpate. *Exp. II*: Twenty-eight male guinea pigs weighing approximately 350 g each were injected subcutaneously with 35 μg of reserpine-H^3 and sacrificed in groups of four after ⅓, 1, 2, 8, 24 and 48 hours. A record was kept of the observable pharmacological responses such as ptosis, myosis and shivering. Brains and livers were removed and analyzed as described above. *Exp. III*: Six male guinea pigs were treated as in Exp. II except that reserpine was labeled with C^{14} in the carboxy carbon of the TMBA moiety. Four of these animals were sacrificed by exsanguination after 48 hours and the remaining two after 72 hours. Livers and brains were removed and extracted as described earlier except that 100 μg of unlabeled reserpine were added as carrier at the time of homogenization of the tissue. The final fraction B was chromatographed on paper(7) and the fluorescent reserpine spot eluted and counted. *Determination of Radioactivity*: Samples obtained from experiments in which animals received 2 mg of reserpine-H^3 intravenously as well as those receiving 35 μg of reserpine-C^{14} were counted in a Tricarb liquid scintillation spectrometer. The remaining samples were combusted(8) and counted in a low-level gas counting system described by Broecker(9) and Giletti(10). This heavily shielded anti-coincidence counter can readily detect 10^{-7} microcuries of H^3 in a sample.

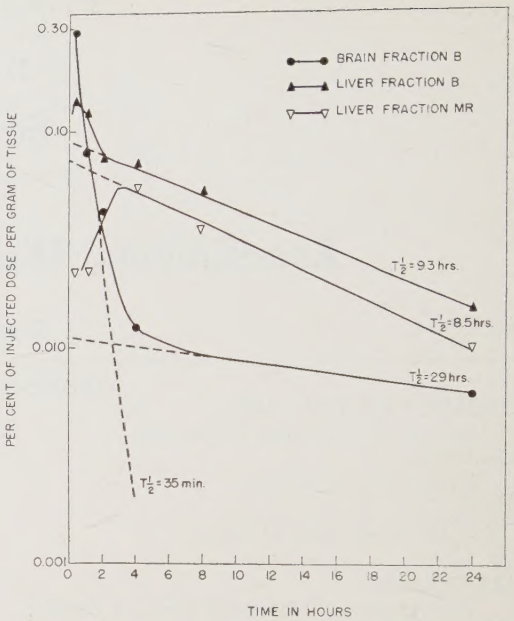


FIG. 1. Percent of inj. tritium recovered in fractions of brain and liver following the intrav. inj. of 2 mg of reserpine-H^3 into guinea pigs.

Results. The percentage of injected tritium found/g of tissue in brain and liver fraction B and liver fraction MR after the intravenous injection of 2 mg of reserpine-H^3 can be found in Fig. 1. No MR could be found in the brain. Maximum tritium concentrations were found within 20 to 30 minutes in brain and liver fraction B, which then decreased rapidly reaching in 2 to 4 hours more stable levels showing biological half-lives of 29 and 9.3 hours respectively. The tritium in liver MR fraction, however, reached its maximum only after 3 to 4 hours but then decreased with a biological half-life of 8.5 hours which was comparable to that for liver fraction B.

Urine and blood tritium levels can be found in Table I. It is observed that the tritium content of plasma fraction B + MR remained relatively constant over the entire experimental period. This was true also for the same fraction of the blood cells but at a very much lower level. The tritium content of fraction C of the plasma was very much lower than that observed for fraction B + MR. No tritium was detectable in fraction C of the blood cells. In urine, however, most of the tritium could be accounted for in fraction C

TABLE I. Blood and Urine Tritium Content following Intravenous Injection of 2 mg of Reserpine-H^3 into Male Guinea Pigs.

Time (hr)	% of inj. dose of 2 mg of reserpine-H^3 found in: Urine fraction B + MR*	Urine fraction C†	Blood plasma fraction B + MR	Blood plasma fraction C	Blood cell fraction B + MR	Blood cell fraction C
⅓			.66	.12	.13	ND
			1.43	.20	.20	"
1			1.00	.08	.14	"
			.80	.21	.17	"
2	.066	2.0	.60	.17	.07	"
			.93	.14	.10	"
4	.38	2.0	1.10	.03	.14	"
	1.26	2.6	1.33	.14	.07	"
8	.45	5.4	.63	.10	.07	"
	.66	5.2	.58	.07	.07	"
24	1.08	4.2	.66	ND	.07	"
0– 5	.22	4.6				
5–24	.76	1.1				

* Fraction B + MR would include tritium derived from reserpine and methyl reserpine.

† Fraction C would include all of the trimethoxybenzoic acid.

ND means none detected.

rather than in fraction B + MR.

The results of Exp. II in Table II show that the tritium content reached a maximum in the brain in 20 minutes and in the liver in 60 minutes after injection and was still detectable at lower levels in fraction B of brain and liver after 48 hours. The rate of decrease in tritium concentration was very much slower compared to that of the experiment utilizing the intravenous route and this was probably the result of the slow absorption of reserpine from the injection site. In spite of the low levels of reserpine administered and the small amounts which entered the tissues, the curves were still biphasic in nature. Evaluation of the pharmacological effects of the single subcutaneous injection of 35 micrograms showed that observable ptosis came on after about 2 hours, increased in intensity and became accompanied by myosis, and shivering, after about 8 hours. The intensity of effect fell off somewhat after 24 hours and was practically gone after 48 hours. Thus, during the period when all observable effects had appeared, reached a maximum and disappeared, the tritium concentration of the brain remained unchanged. It was necessary to be certain that the low levels of tritium being measured were from reserpine and not from stray tritium atoms which had been stripped off of the reserpine by exchange reactions. Another group of guinea pigs were injected with reserpine-C^{14} and sacrificed after 48 and 72 hours. The results of experiment III in Table III showed that approximately 70% of the C^{14} recovered could be accounted for in the spot on the paper chromatogram occupied by the unlabeled reserpine which had been added as carrier at the time of extraction. This was so after 72 hours, when no effect was evident, as well as after 48 hours, when approximately one-half

TABLE II. Concentration of Tritium in Tissue Fraction B and Graded Responses of the Animals following Subcutaneous Injection of 35 μg (Approx. 0.1 mg/kg) of Reserpine-H^3.

Time, hr	Brain % I.D./tissue	Brain μg/g*	Liver % I.D./tissue	Liver μg/g	Inj. site, % I.D./tissue	Graded response†
⅓	.18	.023			30.65	
	.12	.013	.83	.019		
1	.13	.014	1.11	.022	4.77	
	.13	.014				
2	.11	.012	.15	.004		+
	.10	.011				
8	.080	.009				+++
	.080	.009			1.14	
24	.11	.012			.35	++
	.10	.011				
48	.08	.009	.08	.002	.18	±
	.09	.009				

* This column is calculated on the assumption that all of the tritium is contained in reserpine.

† Mild ptosis, +; ptosis plus myosis, ++; marked ptosis, myosis and shivering, +++.

TABLE III. C^{14} Content of Guinea Pig Brains 48 and 72 Hours after Subcutaneous Injection of 35 μg of Reserpine-C^{14} and Its Recovery as Reserpine on a Paper Chromatogram.

Hr after inj.	μg of reserpine/g of brain	% of total C^{14} on paper recovered in reserpine spot
48	.015	78
48	.009	67
48	.012	73
48	.018	75
72	.012	78
72	.010	57

of the animals showed only slight ptosis. These results were true for both brain and liver fraction B. No C^{14} was detectable in the total volume of blood obtainable by exsanguination indicating that the reserpine present in these tissues could not be from the contaminating blood. The levels of C^{14} found in these experiments were quite comparable with the levels of tritium found in the experiments utilizing animals which had received tritiated reserpine.

Discussion. In general the results of the experiment utilizing 2 mg of reserpine-H^3 per animal intravenously agree with those found with similar doses of reserpine-C^{14}. In both cases the concentration *vs.* time curves could be resolved into two components one having a short and the other having a long biological half-life. Attempts were made to account for these two components by examination of subcellular fractions. Trial experiments indicated that while most of the reserpine could be found in the particulate matter of the cell no one fraction could be assigned the unique property of accumulating and retaining reserpine. The biological decay curves for tritium in all of the particulate fractions were resolvable into two components as was the case for the complete homogenate. It should be recognized that the distribution of reserpine found in the subcellular fractions may reflect more of a redistribution after homogenization than the actual situation in the intact cell.

Any attempt to compare directly the urinary excretion values obtained in these experiments with those obtained with reserpine-C^{14} will find that the tritium values are low particularly for fraction C. However, when one considers the fact that only 1/3 of the tritium in the reserpine molecule resides in the TMBA moiety the values become quite comparable.

The tritium content of the plasma B + MR fraction is probably derived mostly from its MR content since in the studies with reserpine-C^{14}(2) and those reported by Hess *et al.*(3) for unlabeled reserpine, blood levels of reserpine quickly drop to very low values. The biological half-life for reserpine in plasma may be calculated from the data of Sheppard *et al.*(2) as being approximately 50 minutes.

It is interesting to note that while the distribution of reserpine between plasma and blood cells is in favor of the plasma, that between plasma and cells of tissues favors the latter. It appears likely that this ability to concentrate reserpine against a concentration gradient is in some way related to its prolonged action.

The presence of what appeared to be two components in the brain and liver fraction B concentration *vs.* time curves suggested the need for studies utilizing much smaller and more pharmacological doses of reserpine. It was felt that the short half-life component might have reflected the entrance and release of a physiological excess of reserpine and therefore might be confusing the picture. The much smaller dose of 35 micrograms/animal was given subcutaneously to allow for a slower onset and development of symptoms. The short half-life component was markedly reduced but its presence was still evident. Though this point is still not fully explained the experiment pointed up the persistent presence of reserpine throughout and beyond the period of observable pharmacological responses. The earlier conclusions that the intensity of the pharmacological effects of reserpine bear no relationship to its concentration in the brain have been strongly reinforced by the experiments reported here. While the paper chromatographic identification of reserpine should not in any way be construed as absolute proof of the nature of the tritiated material present in the brain and liver it is certainly very strong evidence. One may safely state that the material cannot be isoreserpine, reserpine N-oxide, didehydro or

tetradehydro-reserpine since reserpine is satisfactorily separated from these substances. The comparable levels of tritium and C^{14} labeled reserpine found in the livers and brains after 48 hours strongly suggest that practically all of the tritium is biologically stable.

The question as to the site and mechanism of action of reserpine still remains to be answered. The lowering of the levels of serotonin and norepinephrine by reserpine in brain and other tissues observed by a number of workers(11,12,13), while probably offering some hint as to the mechanism of action, fails to explain the time course of events following the administration of this drug. Shore *et al.* (11) have stated that one is not able to correlate the observable effects of reserpine with the concentration of serotonin in the brain of rabbits.

Perhaps it would be of value to look to some tissue other than the brain as the primary site of action of reserpine. One could suggest that initially reserpine acts on some metabolic pathway of a vital organ such as the liver with the result that there occurs a quantitative change in the output of some substance or substances involved in the normal functions of the central nervous system. Such an effect could explain the lag observed in the onset of symptoms of reserpine action. This hypothesis is currently being used as a guide to further work on the mechanism of action of reserpine.

Summary. (1) Reserpine randomly labeled with tritium had a time-concentration relationship in liver and brain similar to that observed for reserpine labeled with C^{14} in the TMBA moiety. (2) Throughout and beyond the entire period of observable pharmacological response, reserpine was found to be present in brain and liver. (3) The concentration of reserpine in the brain varied little during the entire period of observable activity and bore no relationship to the intensity of response. (4) The material present in the brain was indistinguishable from reserpine by paper chromatography.

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Effect of Internal Emitters on Red Cell and Plasma Volumes of Beagle Dogs.* (23857)

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The hematological alterations produced by internally deposited radioisotopes have been studied in this laboratory for several years. The particular isotopes under investigation are plutonium, radium, radiothorium, mesothorium and strontium (Sr^{90}). Among blood changes that have been induced by these bone seeking isotopes, anemia is one of the most frequent. Relatively few studies have been made on anemias induced by internal irradiation as compared to numerous investigations on external irradiation. Our primary object is to ascertain the amount of retained isotope required to induce anemia, and to determine the time of onset, duration and type of anemia. Hematological alterations observed during the first year following administration of plutonium and radium have been reported(1). Absolute changes in red cell and plasma volume determined by Cr^{51} tagged red cells and I^{131} tagged human albumin are presented here.

Methods. The 21 control and 29 isotope treated animals are part of a colony of about 350 pure-bred Beagle dogs. They are housed in modern kennels and constantly under the care of full time veterinarians and their staff who maintain the animals on most optimum dietary conditions. Adult dogs, approximately 16 months of age, are injected intravenously with a single dose of isotope. Dogs bearing retained amounts of 0.27, 0.81 and 2.5 μc/kg of radiothorium and mesothorium and 0.81 and 2.5 μc/kg of plutonium and radium were used. Anemia occurs at these dose levels at one to 6 months post injection with recoveries and relapses at irregular intervals thereafter. Injected animals were studied from one to 45 months post injection and many were re-evaluated at multiple time intervals for total of 50 determinations on 29 dogs. Red cell volumes were measured by Cr^{51} tagging by modification of method by Read(2). Plasma volumes were obtained simultaneously by I^{131} tagged human albumin. About 50 ml of blood were collected in a plastic Fenwall bag containing 10 ml ACD solution, 0.5 to 12 μc Cr^{51} (Rachromate‡) and 0.5 to 1.5 μc I^{131} (RISA‡). After 45 minutes Cr^{51} uptake was stopped with 50 mg sodium ascorbate. Total counts were determined by obtaining an aliquot sample for counting and by weighing the bags before and after transfusion. Specific gravities of blood and plasma were determined with graded solutions of copper sulfate concentrations(3). Three 5 ml samples at 15-minute intervals $\pm$ 10 seconds served as dilution samples. Hematocrits were determined in Wintrobe tubes centrifuged at 3,000 rpm for 30 minutes and were not corrected for trapped plasma. Counting in sealed glass vials in shielded well-type scintillation counter with pulse height analyzer yielded about 90,000 cpm/μc Cr^{51}; 380,000 cpm/μc I^{131}; and 150 cpm background. Blood was placed in weighed vials (nearest 0.1 mg), centrifuged; plasma was withdrawn into weighed vials to be counted separately. Counts from packed cells were corrected for small amount of remaining plasma. From 15% to 50% of counts in the vial containing red cells represented remaining plasma. From 5% to 20% of counts in plasma were from Cr^{51} which did not tag red cells. Three points at 15-minute intervals were plotted on semi-logarithm graphs and the best fit line extrapolated to 0 time for plasma volume. The majority of determinations showed little scatter and constant slope. Red cell volume was calculated from the 30-minute dilution sample.

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‡ Abbott Laboratories.

TABLE I. Comparison of Blood Volume Values in Control and Isotope Treated Beagle Dogs.

Group	No. of dogs	RBC vol (ml/kg)	Plasma vol (ml/kg)	Blood vol (ml/kg)	Venous hematocrit	Total body hematocrit	Hematocrit ratio
Control	21	43.0 ± 9.5*	51.4 ± 12.4	94.4 ± 16.6	46.4 ± 4.4	45.7 ± 6.8	.99 ± .09
Isotope treated (non-anemic)	11	43.3 ± 12.6	61.9 ± 8.7	105.2 ± 17.2	40.6 ± 5.1	40.7 ± 6.1	1.02 ± .21
Isotope treated (anemic)	22	24.4 ± 5.7	53.3 ± 11.1	78.7 ± 14.5	36.5 ± 5.3	32.1 ± 5.2	.88 ± .10

* Means are expressed ± stand. dev.

Results. The precision of this method was evaluated by making 2 determinations at one-week intervals in 10 dogs. Mean variation in red cell volume was 10% (0.5% to 19.2%) and in plasma volume 7.7% (0.2% to 16.3%). Red cells and plasma were counted immediately prior to second determination to correct for residual Cr^{51} and I^{131}. In 4 animals plasma volume was determined alone with I^{131} and then repeated 2 days later in combination with red cell volume. Mean variation was 6% (2.0% to 11.9%).

In Table I the animals are divided into 3 groups: 1) controls, 2) isotope treated dogs in non-anemic phase, and 3) isotope treated anemic dogs. An animal was considered anemic when red cell volume was below the range of control values (<33.4 ml/kg). Blood volumes are expressed per kg of dog's average weight. In last column of Table the venous hematocrit is divided by total body hematocrit (red cell vol/total blood vol) and expressed as ratio (hematocrit ratio). This ratio expresses relative compartmentalization of red cells in large veins to body pool. There were 16 determinations on 11 non-anemic dogs and 34 determinations on 22 anemic animals.

Isotope treated dogs with decreased red cell volumes had normal plasma volumes. Only one severely anemic radiothorium dog (red cell vol 10.1 ml/kg) had a slightly decreased plasma volume of 35.2 ml/kg. Total blood volumes and hematocrit ratios in this anemic group were decreased from control values (t-test differences with $p = .001$). Blood volume values on isotope treated non-anemic dogs were generally similar to control animals.

Table II gives correlation coefficients between red cell volumes and hematocrit ratios. There is a good positive correlation in injected group (includes both anemic and non-anemic dogs) and a poor correlation in control group.

Red cell volumes and venous hematocrit values are correlated in Table III. There is good correlation in isotope treated anemic dogs in contrast to poor correlation in control and isotope treated non-anemic groups. In this Table the control group includes red cell volume and hematocrit values from 29 dogs previously measured without plasma volume determinations.

Discussion. A method is presented for simultaneous determination of red cell and plasma volume by isotope technics. A varia-

TABLE II. Correlation between Red Cell Volume and Hematocrit Ratio.

Group	RBC vol, mean	Hematocrit ratio, mean	No. determinations	Correl. coeff., r	P value
Control	43.0	.99	21	.429	.1–.05
Isotope treated	31.1	.93	50	.712	.001

TABLE III. Correlation between Red Cell Volume and Venous Hematocrit.

Group	RBC vol, mean	Ht, mean	No. determinations	Correl. coeff., r	P value
Control	42.8	48.6	52	.369	.01
Isotope treated (non-anemic)	40.5	45.8	32	.354	.05
Isotope treated (anemic)	25.8	38.9	46	.690	.001

tion of this method has been reported by others both as a simultaneous technic(4) and as a sequential technic employing Cr^{51} immediately following I^{131} plasma volume determination(5). Drawbacks to the method are errors introduced by inability to separate I^{131} and Cr^{51} in the counting device. In counting red cells correction must be made for contaminated I^{131}. A small amount of Cr^{51}, which cannot be corrected for, contaminates the plasma. Calculations from data published by Reeve *et al.*(6) demonstrate in 3 dogs, listed with repeat P^{32} red cell volume and T-1824 plasma volume, a variation of 9.1% to 18.3% and 0% to 8.8% respectively. These are very similar to variations seen here. Plasma volume determined alone differs only slightly from that determined simultaneously (average variation 6%). The red cell volume of control group (average $= 43.0 \pm 2.1$), is almost identical to a group of 29 dogs in which red cell volume was determined alone (average $= 42.6 \pm 1.7$). The method presented here offers advantages of gravimetric measurements, a single injection of isotope mixture, and concomitant plasma and red cell volumes. Experimentally the technic is reasonably reproducible and accurate.

From Table I it may be seen that in control dogs the mean venous hematocrit value is similar to total body or true hematocrit value with a hematocrit ratio approaching unity. However, in control dogs there is a poor correlation between red cell volume and venous hematocrit and hematocrit ratio (Tables II and III). Thus, the venous hematocrit does not reflect accurately changes in red cell volume. On the other hand, anemic isotope treated dogs have a higher venous hematocrit than total body hematocrit (different by T test of .002 level), and the venous hematocrit reflects fairly accurately changes in red cell volume (Table III). In these anemic dogs there is a striking contrast between reduction of venous hematocrit (80% of control) and decrease in red cell volume (60% of control). A decreased hematocrit ratio has been noted in people in various disease states, *i.e.*, chronic anemia, acute hemorrhage, heart failure, dehydration, hypoproteinemia, post operative shock(7); although some studies reveal a quite constant ratio over a wide hematocrit range(8).

These results suggest that when isotope treated dogs become anemic, the venous red cell compartment as represented by venous hematocrit increases with no accompanying change in plasma volume, and the red cell pool falls elsewhere. In other words, there is a relative shift of red cell mass from other areas into the venous pool. The red cells could come from the splenic pool or capillary bed.

Spleens of normal dogs can store from 2 to 17% of red cell volume depending on degree of contraction or relaxation(6). Splenectomy reduces this wide fluctuation(6). The splenic pool could become depleted in isotope treated dogs, and this has been borne out at autopsy as some dogs have had small atrophic spleens.§ About 17% of total blood volume is found in minute vessels of the dog(9). Preliminary study of post mortem sections suggests that there is a decreased capillary bed in many isotope treated dogs. Further study is necessary to determine the main source of additional red cells in larger veins.

Summary. Simultaneous red cell and plasma volumes have been measured with Cr^{51} and I^{131} in control beagle dogs and dogs with internally deposited radioisotopes. The reliability of the method is discussed. In isotope treated anemic dogs the venous hematocrit tended to be higher than the total body or true hematocrit but more accurately reflected red cell volume changes than in controls. Plasma volume did not change to account for this relative increase in venous hematocrit. It is postulated that this relatively high venous hematocrit probably represents a shift of red cells into large caliber veins.

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Production of Experimental Cardiac Hypertrophy in Rats by Reduced Atmospheric Pressure and Elevated Environmental Temperature.* (23858)

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In our investigations on proteins of the heart a need arose for production of graded cardiac hypertrophy in the rat. It was also necessary that the cardiac changes be related entirely to increase in work load. Use of an aorta or renal artery tie yielded erratic results. The possible occurrence of secondary cardiac pathology due to renal necrosis made these technics undesirable. Hypertrophy has been reported frequently following exposure to discontinuous hypoxia. Van Liere and Feder (1) observed 24% increase in relative heart weight following exposure of rats to atmospheric pressure of 303 mm Hg, 4 hours a day for 41 days. This technic seemed to offer many advantages and it was employed along with elevated environmental temperature. It was possible by this means to produce graded hypertrophy of any desired degree. For example a 50% increase in heart weight occurred after approximately 12 days of treatment.

Methods. Rats of the Addis-Slonaker strain maintained on stock diet were used. The weight range was 135-165 g. They were daily exposed (including weekends) for 4 hours to 37°C ± 1°C and an elevation of 25,000 ± 1,000 ft. in an evacuation apparatus.‡ When heart weights were to be obtained, the rat was killed on the morning following last exposure and the heart was freed of the great vessels and both atria. The chambers were cut and blood removed as completely as possible by tapping on filter paper. Growth did not occur during period of treatment; the animals either gained or lost a few grams over the starting weight. If an occasional rat lost more than 6 g, it was discarded.

Results. The findings were documented as regression of log of relative heart weight (R) on accumulated exposure time (T) (Fig. 1). R is the ratio of observed heart weight/predicted heart weight. For predicted heart weight the equation of Addis and Gray(2) was used. This had been derived for animals of this strain. R for untreated animals is approximately .96 rather than 1.00 since the Addis-Gray equation is based upon the intact heart. Since a sex difference was not observed, the data of males and females were pooled.

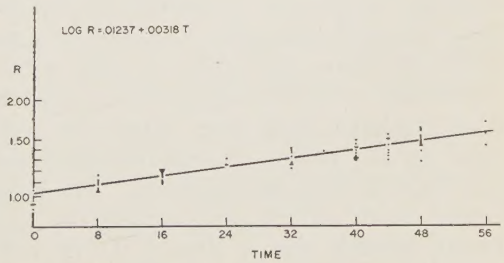


FIG. 1. Accumulated exposure time (T) and heart wt observed/heart wt expected (R). T = time in hr. n = 75. Correlation coef. = .83. z transformation yielded $\frac{z}{\sigma_z} = 10$. t = 12.7. V = .00144.

* Supported by grant from Am. Heart Assn. and Public Health Service.

† Investigator, Howard Hughes Fn.

‡ The authors are grateful to Dr. Benjamin H. Ershoff for loan of this equipment.

It may be seen that a 50% increase in heart weight occurs after approximately 12 days of treatment. Van Liere and Feder obtained an increase in heart weight of only 24% after 41 days of treatment employing reduced atmospheric pressure alone.

Summary. Graded cardiac hypertrophy has been produced in the albino rat following exposure to 37°C ± 1°C and 25,000 ± 1,000 ft. for 4 hours a day. After 12 days of treatment a 50% increase in heart weight is produced by this means.

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Purification of Human Leukocytes.* (23859)

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Attracted by availability of human white blood cells (WBC) as compared to other cellular elements of body, numerous investigators have utilized a variety of methods for leukocyte isolation. Most technics for separating white cells from whole blood have emphasized leukocyte yields. However, as more exacting measurements are required, it becomes increasingly important to obtain preparations which are free of other formed elements. In these situations purity rather than yield must be the criterion for evaluating the merit of an isolation procedure. Of many technics we examined, the most promising for obtaining high white cell purity were fibrinogen sedimentation method of Buckley, Powell, and Gibson (1) as modified by Skoog and Beck(2) and the phytohemagglutinin method of Li and Osgood(3). The present report summarizes our experience with these 2 methods and introduces several modifications to facilitate evaluation and to increase purity of white cells.

Methods. Phytohemagglutinin prepared from McCaslan Pole Bean (*Phaseolus vulgaris*) by the method of Boyd(4)† was used and found to be a panagglutinin. The bean extract, at its optimal concentration for leukocyte isolation, has a titer of 1/256 with fresh erythrocytes of varying blood group and Rh types. Venous blood is collected with a few drops of potassium oxalate solution serving as anticoagulant. All syringes, pipettes, and other glassware are siliconed. The bean extract (0.3 ml) is added to 10 ml blood in a 15 x 100 mm test tube at room temperature. The contents are mixed by inversion, and the glass surface above blood is cleaned with cotton swab. The tubes are centrifuged (International Size 1, Type C centrifuge, Head No. 246 or International Clinical Centrifuge, Model CL) at 50 g for 4 min. The supernatant is removed and centrifuged at 100 g for 10 min. to sediment the leukocytes. The WBC are resuspended in 5-10 ml of phosphate buffer. This buffer is prepared from 2 g sodium acetate, 0.003 g ascorbic acid, and 1.42 ml 85% phosphoric acid; the mixture is diluted to one liter after adjusting to pH 7.45 with NaOH. (Tullis(5) pointed out the advantages of acetate and ascorbate.) The cell suspension is centrifuged at 90 g for 7 min., and the final button is resuspended in the same phosphate buffer. With experience, this procedure from time of collection to preparation of final phosphate suspension should require 45-60 min. WBC counts are prepared immediately using Toisson's solution as diluting fluid. (Toisson's solution is composed of 20 ml glycerin, 160 ml distilled water, 8 g sodium sulfate, 1 g calcium chloride, and 0.025 g methyl violet.) Slides for differential leukocyte counts are prepared by adding a

* This research was supported in part by an N.I.H. grant.

† Phytohemagglutinin was a gift from Dr. C. W. Cotterman.

TABLE I. Evaluation of Leukocyte Purification by the Phytohemagglutinin Technic in Conjunction with the Use of Phosphate Buffer.

	No. determinations	WBC recovery		WBC purity		
		Mean %	S.E.	% of cell population Mean	S.E.	No. WBC/No. RBC (mean)
Supernatant plasma						
Normal	10	30.9	3.22	72.7	5.64	2.7
Leukemia	5	44.8	6.76	96.5	1.48	27.6
After PO_4 treatment						
Normal	12	18.5	1.30	93.8	1.95	15.1
Leukemia	20	38.4*	7.66	98.8	.48	82.3

* Data available from 12 determinations.

drop of 8% albumin to a drop of phosphate suspended WBC. The method of Skoog and Beck(2) for isolating WBC by sedimenting erythrocytes with fibrinogen was employed.

Results. With the phytohemagglutinin technic leukocyte purity has been evaluated after initial removal of erythrocytes when WBC were still suspended in their own plasma and again after treatment with phosphate buffer. Results summarized in Table I show high purity leukocyte suspensions in the supernatant plasma. With the elevated white cell counts of leukemic bloods higher WBC purity (96.5%) was obtained at this point in procedure than with normal bloods (72.7%). Utilizing the differential lytic effect of phosphate buffer, WBC purity was increased to 93.8% and 98.8% for normal and leukemic bloods, respectively. After phosphate treatment, 31 of 32 preparations exhibited cell populations that were at least 90% leukocytes. Chi-square analysis (χ^2) of differential counts revealed no significant variation in distribution of WBC types in whole blood compared to phosphate suspensions of isolated cells.

The results of applying fibrinogen sedimentation of erythrocytes to isolation of leukocytes are presented in Table II. In each of 5 instances decided improvements in purity were obtained by washing isolated cells with phosphate buffer. Before treatment, the cell populations had a mean leukocyte content of 25.9% (WBC : RBC = 0.35); after treatment, 82% (WBC : RBC = 4.56).

Discussion. Several criteria have been employed for evaluating the merit of some 10-15 leukocyte separation procedures. We have insisted that leukocytes be essentially free of erythrocytes, that white cell yield be adequate and that the cells be morphologically intact and freely suspendable for counting.

The phytohemagglutinin technic(3) has been superior to other methods. Several modifications have been presented to facilitate evaluation and to improve further the purity of WBC. Use of Toisson's solution for staining white cells has simplified differentiating leukocytes from erythrocytes in the counting chamber under unusual circumstances where white cells greatly outnumber the red. In preparation of slides for differential counts on isolated leukocytes, it was advantageous to suspend the cells in albumin. In aqueous medium they are frequently ruptured when smeared on the slide. That distribution of

TABLE II. Leukocyte Recovery and Purity following Fibrinogen Sedimentation of Erythrocytes of Normal Whole Blood.

No. determinations	Vol fibrinogen/vol blood	Fibrinogen conc. (g/100 ml)	WBC recovery		WBC purity		
			Mean %	S.E.	% of cell population Mean	S.E.	No. WBC/No. RBC (mean)
2	2	4			30.8	21.65	.45
3	1	4	65.0	9.34	29.7	13.76	.42
4	1	6	57.2	3.97	21.1	4.96	.27
3	.5	4	57.8	10.22	35.5	16.61	.55
2	.5	6	40.5	8.50	17.1	4.80	.21

WBC types is the same after isolation as in whole blood argues strongly that the cells are a representative rather than a resistant population. In this study yields have been sacrificed for the sake of purity. Leukocyte purities greater than 90% have been uniformly achieved by washing and resuspending isolated WBC in a phosphate buffer which lyses residual red cells while leaving white cells intact. This result is considerably better than the optimal 50% purity recorded by Skoog and Beck(2) for the fibrinogen technic or the 52.5% and 83.8% for normal and leukemic bloods, respectively, calculated from data of Li and Osgood(3). The phytohemagglutination technic with phosphate buffer is recommended to those workers who require white cell suspensions of high purity.

Summary. Methods for preparing human leukocytes from venous blood have been investigated. Sedimentation of erythrocytes by fibrinogen or phytohemagglutinin of *Phaseolus vulgaris* have given best results. With white cell purity as a goal, the phytohemagglutination method has been clearly superior, giving preparations of 72.7% and 96.5% from normal and leukemic bloods, respectively. These values have been increased to 93.8% and 98.8% by utilization of a phosphate buffer which preferentially lyses red cells while leaving white cells intact.

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Effect of Divalent Cations on Proteolysis of Fibrinogen.* (23860)

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Calcium, in conjunction with an unidentified serum factor, exerts an effect on the fibrinogen-fibrin conversion such that the final clot differs significantly from that formed in the absence of one or both of these components. The clots differ in solubility in dilute acid(1), in concentrated urea(2), and in concentrated lithium bromide(3). They differ also in tensile strength(4) and in ease of conversion from α- to β-keratin structures, as demonstrated by x-ray diffraction patterns(5). It has also been demonstrated(6) that the intermediate polymers formed in the conversion are longer and that the rate and extent of their dissociation is diminished in the presence of Ca. Recently, Thomas† observed the inhibition by Ca of streptokinase-induced clot lysis. The conditions of the experiment were such that the effect of Ca could have been on the nature of the clot formed: Ca does not inhibit activity of plasmin(7).

Since these effects might have a common origin in the initial proteolytic attack of thrombin on fibrinogen, it appeared of interest to investigate the effect of Ca on hydrolysis of fibrinogen by trypsin. Both thrombin(8) and plasmin, the streptokinase-induced fibrinolysin(9), possess proteolytic specificity similar to trypsin. Chymotrypsin, a proteolytic enzyme of differing specificity, was studied for comparison.

Methods and materials. Fibrinogen, 90-94% clottable by the method of Laki(10), was prepared from bovine plasma fraction I‡ by the methods of Morrison(11) and Laki (12). Other reagents were obtained commercially. The assay was similar to that of Kunitz(13). A 0.9 ml aliquot of a solution

* This study was supported in part by USPHS grant, H-1968(C3).

† W. A. Thomas, personal communication.

‡ Generously supplied by Armour Laboratories, Chicago, Ill.

TABLE I. Effect of Various Divalent Cations on Proteolysis of Fibrinogen by Trypsin.

Substance added	Increase, OD_{280}
.005 M EDTA	.355
Pyrophosphate	.365
.001 M $CaCl_2$	.225
$MgCl_2$	.240
$CoCl_2$	.237
$ZnCl_2$	.255
$MnCl_2$	.233
$CdCl_2$	.225

of fibrinogen in 0.05 M Tris§ buffer, pH 7.4, containing sufficient NaCl to bring the final ionic strength to 0.40, was warmed in a constant temperature bath at 38°C. One-tenth ml trypsin (1.0 mg/ml) or chymotrypsin (0.2 mg/ml) was added with shaking. After 20 minutes 1.0 ml 5% trichloroacetic acid was added, the mixture was allowed to stand for one hour, centrifuged for 20 minutes, and the optical density of the supernatant was measured at 280 mμ on the Beckman Model DU spectrophotometer. Appropriate blank determinations were run with addition of trichloroacetic acid before addition of the enzyme. Trypsin autolysis did not measurably contribute to the optical density of the supernatant. A preliminary experiment indicated that the increase in optical density was approximately linear in the time interval 0-30 minutes, and the 20-minute incubation period was chosen for convenience as a measure of the initial rate of proteolysis.

Results. Table I demonstrates that pyrophosphate, which would also be expected to reduce the activity of Ca ions, is effective as EDTA in enhancing the tryptic digestion of fibrinogen. This table also indicates that the other divalent cations tested were approximately equal to Ca in effectiveness. It was found that the rate of proteolysis without addition of chelating agent or metal ion was variable from one preparation to another, indicating contamination by traces of metals. The rates of some preparations approached that obtained in the presence of chelating agents.

In Table II, the averages of determinations at various Ca concentrations are expressed as percentage maximum initial rate. It is apparent that the rate of proteolysis is diminished at very low Ca concentrations, and that 50% reduction occurs at about 10^{-3} M.

§ Sigma Chemical Co.'s trishydroxymethylaminomethane.

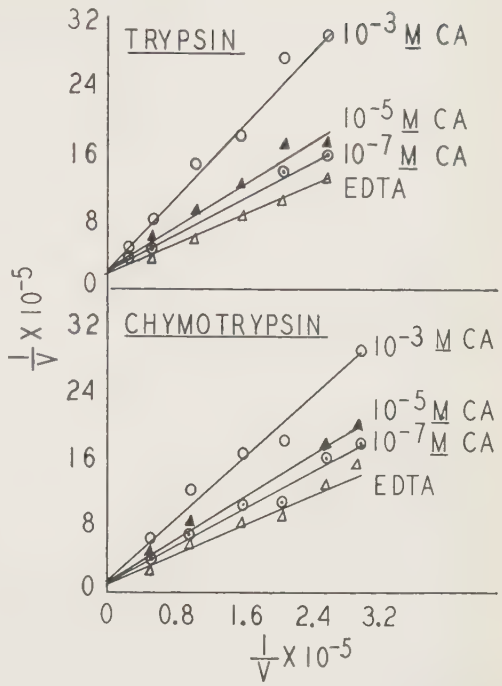


FIG. 1. Lineweaver-Burk reciprocal plot of effect of various concentrations of calcium on kinetics of proteolysis of fibrinogen by trypsin (0.1 mg/ml final conc.) and by chymotrypsin (0.02 mg/ml final conc.).

The results of varying fibrinogen concentration are expressed in Fig. 1 in a Lineweaver-Burk reciprocal plot. The kinetic curves are typical of a competitive inhibition.

Discussion. Since maximal rates of proteolysis were obtained in the presence of pyrophosphate as well as with EDTA, it would appear that the ability of these anions to enhance proteolysis of fibrinogen lies in their Ca-binding capacity rather than in a specific

TABLE II. Comparison of Effects of Varying Concentrations of Calcium on Rates of Tryptic and Chymotryptic Digestion of Fibrinogen, Expressed as Percentage of Maximum Initial Rate.*

Substance added	% max initial rate	
	Trypsin	Chymotrypsin
.005 M EDTA	100	100
10^{-7} M $CaCl_2$	79.3 ± 5.1	87.7 ± 5.4
10^{-5} M "	74.6 ± 3.8	77.6 ± 2.6
10^{-3} M "	49.8 ± 6.8	54.0 ± 1.3

* The exp. errors are expressed as stand. dev.

interaction with the protein. The only other anion present in the test system was chloride.

The apparent competitive inhibition of the reaction could most simply be explained by a competition between Ca and substrate for reactive sites on the enzyme. However, it has been demonstrated that Ca does not inhibit the activity of trypsin on synthetic substrates, on casein, or on denatured hemoglobin; rather, it has been shown to stabilize the enzyme(14). It has been further shown that Ca enhances the activity of chymotrypsin for these substrates(15). Since Ca inhibits the proteolysis of fibrinogen by both of these enzymes, it may be inferred that the action of Ca is not upon the enzyme but is to stabilize the substrate.

Gorini and co-workers(16) have demonstrated a similar effect of Ca and Mn on the tryptic digestion of serum albumin and lysozyme and have correlated this effect with the ability of those cations to decrease the rate of thermal denaturation of those proteins. They suggest that the metal exerts its effect upon an equilibrium between 2 forms of the protein, only one of which is susceptible, at least rapidly, to proteolytic action or to irreversible thermal denaturation. This concept follows from the suggestion by Linderstrøm-Lang(17) that the primary step in proteolysis is a reversible denaturation.

In view of the concepts of Linderstrøm-Lang and of Gorini, it is suggested that the role of Ca is to shift an equilibrium between two forms of fibrinogen, only one of which is rapidly attacked by the enzyme. The other form is attacked relatively slowly or not at all by the enzyme, but by reversibly combining with the active site(s) serves, in effect, as a competitive inhibitor of the reaction.

There is ample precedent for the concept of reversible conformation changes of proteins in solution(18), and it is not unlikely that the role of the divalent cation is to stabilize the tertiary structure of fibrinogen.

Summary. The effects of various divalent cations on proteolysis of fibrinogen by trypsin and chymotrypsin were studied. Calcium, Mg, Mn, Co, Zn, and Cd were approximately equally effective in reducing the rate of proteolysis by trypsin. At levels as low as 10^{-7} M, Ca began to decrease the rate of proteolysis by either trypsin or chymotrypsin, and a 50% reduction of the rate occurred at about 10^{-3} M. The effects of Ca on both tryptic and chymotryptic digestion appeared to yield the kinetics of a competitive inhibition; and the suggestion is made that Ca acts to affect an equilibrium between two forms of fibrinogen, the one susceptible, the other inhibitory, to proteolysis.

I thank Drs. W. S. Hartroft, W. A. Thomas, and R. M. O'Neal (Pathology Department) and Dr. S. F. Velick (Biochemistry Dept.), Washington Univ. School of Medicine, for stimulating discussions and helpful encouragement.

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Activity of Thyroid Gland During Fasting and Realimentation with Diets High in Carbohydrate.* (23861)

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When normal dogs are subjected to a prolonged fast the blood pressure and pulse rate decline to low stable fasting levels which remain quite constant for prolonged periods(1). If they are realimented with a diet high in carbohydrate fed at the luxus consumption level of 120 cal/M^2/hr, they develop a high systolic pressure and rapid pulse with a low or normal diastolic pressure which will usually persist as long as the diet is eaten(2). Since the latter hemodynamic pattern is similar to that seen in hyperthyroidism and can be produced in normal dogs by feeding thyroid powder, the present studies were undertaken to determine whether increased thyroid activity is the causative factor.

Methods. Thyroidectomy with preservation of the parathyroid glands in dogs, often leaves thyroid remnants, hence, this method of approach is uncertain. For this reason it was decided to determine the activity of the thyroid gland by serum protein bound iodine and I^{131} uptake. Six adult normal dogs (2 males and 4 females) were studied. Two of the animals (1 male and 1 female) were trained and standardized for blood pressure studies. Protein bound iodine was determined by the Leffler method as modified by Reals, Townsend, and Danielson(3,4). At the suggestion of Reals, the method was modified by precipitation of the serum protein with 10% perchloric acid (70-72%) instead of trichloracetic acid. Radioactive iodine uptake was determined in the usual way 24 hours after intravenous injection of 10 mc of I^{131}.‡ Blood pressure was determined by the auscultatory method of Allen(5) with certain modifications and improvements(1,6). *Dietary procedures.* During the control and thyroid feeding periods each dog was fed the usual kennel diet of Nutrena dog food. The fasting period, water *ad libitum,* lasted for 29 days on the 2 dogs on which blood pressure was studied and for 50 days on the remaining 4 dogs. Following the fast the animals were all realimented with a diet high in carbohydrate consisting of cracker meal or boiled white rice§ moistened and flavored with Difco Beef Extract and fed at the luxus consumption level of 120 cal/M^2/hr. One tablespoon of Brewers yeast was added to the diet and one multivitamin tablet‖ was administered daily. After the 2 blood pressure dogs had recovered from the dietary experiments there were given 0.9 g/kg/day of USP thyroid powder #58, mixed with the kennel diet for 16 days. Eleven to 12 determinations of PBI were done on each of the 6 dogs during the control period and from 6 to 10 determinations during the fasting and high carbohydrate diet periods respectively. On the blood pressure dogs 8 determinations were done during the period of thyroid powder administration. On 4 dogs not used for blood pressure studies I^{131} uptake determinations were done as follows: on the control diet the day before beginning the fast; on 50th or last day of fasting and on 28th day of high carbohydrate diet.

Results. (A) *Cardiovascular studies.* The figure shows 2 experiments on the same dog. (A) shows the values for blood pressure and heart rate during the control, fasting and realimentation periods. The high systolic pressure with a rapid pulse and low or normal diastolic pressure caused by realimentation

* Financed by Nat. Heart Inst.; Am. Heart Assn. and Nebraska Heart Assn.

† Present address: Mayo Foundation, Rochester, Minn.

‡ We are indebted to Dr. Arthur T. Tuma of Radioisotope Laboratory of St. Catherine's Hospital for these determinations.

§ Rice and cracker meal have the same hemodynamic effect when fed after long preliminary fast.

‖ Multicebrin (Lilly) contains daily human requirement of main vitamins.

TABLE I. Activity of Thyroid Gland during Fasting and Realimentation with Luxus Consumption High Carbohydrate Diets.

	Protein bound iodine, u/100 cc						Radioactive iodine uptake, %				
Dog	Control	Fasting*			L C carbohydrate*			Control	Fasting†		L C carbohydrate†	
I	2.9	3.4	+.5	$P > .1$	1.7	−1.2	$P < .05$	12	9	−3	15	+3
II	3.3	2.6	−.7	$P = .1$	2.2	−1.1	P "	9	13	+4	18	+9
III	3.4	3.4	0		2.0	−1.4	$P < .02$	5	14	+9	14	+9
IV	3.1	2.9	−.2	$P > .1$	2.1	−1.0	$P > .05$	11	6	−5	14	+3
V	2.2	2.8	+.6	$P > .05$	3.1	+.9	$P > .1$					
VI	3.0	3.1	−.1	$P > .1$	1.6	−1.4	$P < .1$					
Mean	2.98	3.03			2.12							

* Difference from control mean and significance of the difference.
† Difference from control mean.
Blood pressure studies were done on dogs V and VI.

with the high carbohydrate diet is a characteristic response. (B) shows the cardiovascular response to the feeding of thyroid powder. The dietary and hormonal responses are qualitatively and quantitatively very similar and both disappeared promptly when the causative agents were discontinued. In the dietary experiments the PBI values ranged between 2 and 3 μg/100 cc while during thyroid feeding the values were consistently above 15 μg/100 cc. When thyroid feeding was discontinued the PBI values remained above the control level for two weeks while the systolic pressure and heart rate returned to normal in 5 days.

(B) *Protein bound iodine studies.* The Table shows the mean values on each of the 6 dogs during the control, fasting and high carbohydrate diet periods. During fasting the values decreased in 3 dogs, remained the same in one and increased in 2. The probability of the differences from the control values was calculated from t and none was significant at the 5% level of confidence. During realimentation with the high carbohydrate diet, the mean values decreased in 5 dogs and increased in 1. The decreases below the control values were significant at the 5% level of confidence or less in dogs I, II, III and VI but not in dog IV. The increase in dog V was not significant.

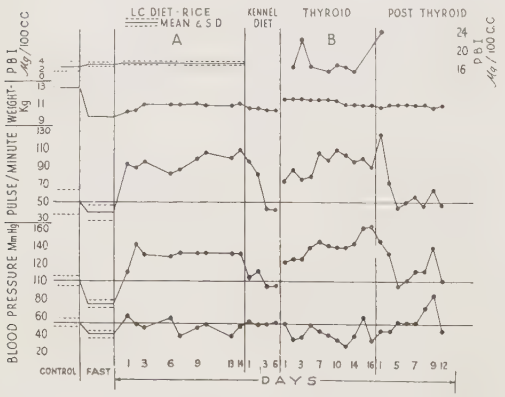


FIG. 1. Blood pressure studies on Dog V.* (A) Effect of realimentation with boiled white rice after fast of 29 days. Fasting values are the mean of last 5 days which represent low stable fasting level. (B) Effect of thyroid powder.

* Similar studies on Dog VI gave identical results.

(C) *Radioactive iodine uptake.* During fasting there was an increase above the control value in 2 dogs and a decrease in the other 2 dogs, but the differences which range from −3 to +9% were not considered sufficiently large to be significant. During realimentation with the high carbohydrate diet there was an increased uptake in all 4 dogs but the magnitude of the increases (+3 to +9% was considered too small to be significant.¶

Summary and conclusions. 1) Activity of thyroid glands of dogs as determined by serum PBI and I^{131} uptake is not significantly altered during prolonged fasting or realimentation with diets high in carbohydrate. 2) The high systolic pressure and rapid pulse with low or normal diastolic pressure which is produced by a diet high in carbohydrate fed at the luxus consumption level of 120 cal/M^2/

¶ Dr. Richard E. Ogborn of Radioisotope Laboratory of Omaha Vet. Adm. Hospital examined the data and agreed that the changes were not significant.

hr after a prolonged fast is strikingly similar to the hemodynamic effect of thyroid powder but it is not due to increased thyroid activity according to the indices studied.

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Chronic Latent Infections of Birds with Western Equine Encephalomyelitis Virus.* (23862)

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(Introduced by K. F. Meyer)
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The endemicity of arthropod-borne viruses such as Western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) in temperate areas indicates the probable existence of a long-term arthropod or vertebrate reservoir of infection. Past studies have indicated that both avian hosts and mosquito vectors are essential in the summer transmission of many of these viruses. This paper reports the finding that WEE virus may persist as a chronic latent infection in experimentally infected birds and the epidemiological significance of this observation.

Materials and methods. Wild birds of several species were trapped in the fall and winter of 1953 to 1956, leg banded with identifying numbers, and held in a mosquito-proof outdoor aviary for the duration of the experiments. A pre-inoculation blood sample for antibody determination was obtained by jugular puncture, and within a few days each bird was inoculated subcutaneously with 0.1 or 0.2 ml of 100 to 15,800 mouse LD_{50} concentrations of BFS 1034, BFS 1703 or F 199 strains of WEE virus. The strains were originally isolated from naturally infected mosquitoes or mites and had been through 3 or 4 serial mouse brain passages. One or more post-inoculation blood samples were taken at intervals of 1 to 5 months, and if possible a final blood sample was collected at autopsy. All blood samples were drawn by jugular puncture into a syringe containing heparin diluted in sterile phosphate buffer saline (pH 7.4). The concentration of heparin was 20 U.S.P. units per ml, and for each ml of blood 0.2 ml of heparin solution was used. Blood plasma was tested for neutralizing antibodies to WEE virus, and as a control for SLE neutralizing antibodies when sample size permitted. The neutralization tests were made in 21-day-old mice with a level of 50 MLD_{50} of virus, and when sufficient plasma was available a second level of 500 MLD_{50} of virus. Neutralization indices above 50 were considered positive, 10 to 49 equivocal and below 10 negative. Birds were subjected to autopsy when found dead in the aviary or when sacrificed by bleeding out. When birds remained in good health, they were held for a minimum of 6 months after infection and a portion was then sacrificed for sampling at intervals extending to 10 months post-inoculation. At autopsy separate sterile instruments were used for each bird but not for each organ sample. From each bird the following tissue samples were usually taken: brain, heart, kidney, spleen, lung, liver, lymph and whole heparinized blood. Occasionally fewer or additional sam-

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ples were taken. Each tissue sample was placed in a separate sterile tube which was sealed in a gas flame and stored in dry ice until tested for virus.

Virus tests were made by inoculating 1:2 to 1:5 dilutions of blood samples and approximately 10% emulsions of organs into 21-day-old mice, 10-day-old embryonated chicken eggs, or chick embryo tissue culture. Organ emulsions were prepared by grinding the organs in a mortar with a diluent of 10% rabbit serum in beef heart broth, pH 7.4, containing 625 units of penicillin and 500 μg of streptomycin/ml. Suspensions were centrifuged at room temperature for 15 to 20 minutes at 2,500 rpm and the supernatant fluid used for inoculation. Each mouse was inoculated intracerebrally with 0.03 ml and intraperitoneally with 0.09 ml of a sample. "Chick embryos" were inoculated with 0.1 ml by the amniotic route. In the last year of study, tissue culture tests similar to those described by Welsh *et al.*(1) replaced the embryonated egg test. Trypsinized cell suspensions of 8 to 11-day-old embryonated eggs were cultured in slanted stationary tubes. The test samples were inoculated in 0.1 ml amounts into the tubes immediately after the addition of the cell suspension. After incubation for 5 days at 37°C, cultures were sub-passed for further establishment of the isolation as well as for confirmation of the absence of a cytopathogenic virus. Any mouse exhibiting signs of encephalitis and sluggish or dead embryos were sacrificed and material was passed for establishment of isolations. The viruses isolated were identified by mouse or tissue culture neutralization tests with specific immune sera. When quantities of the original organ suspensions of positive samples were sufficient, repeat isolations were attempted.

Results. From November 1953 through August 1956, 284 birds survived to be autopsied and tested for virus one month or more after WEE inoculations. The 284 birds were distributed among 9 species as follows: 98 tricolored blackbirds (*Agelaius tricolor*), 18 red-winged blackbirds (*Agelaius phoeniceus*), 24 Brewer's blackbirds (*Euphagus cyanocephalus*), 8 yellow-headed blackbirds (*Xanthocephalus xanthocephalus*), 69 cowbirds (*Molothrus ater obscurus* and *M. ater ater*), 2 Chinese spotted doves (*Streptopelia c. chinensis*), 41 house finches (*Carpodacus mexicanus frontalis*) and 24 English sparrows (*Passer d. domesticus*).

Data from isolations from 1 or more organs of 8 birds at intervals of 1 to 10 months postinoculation are summarized in Table I. Little significance can be given to the predominance of findings in male birds, as the majority, 192 of 284 studied, were males. A wide range of organs was tested in the anticipation that 1 or 2 might be found to be the primary sites of latent infection. There is no evidence of such localization, as 6 different tissues were positive in the 8 birds. The finding of positive blood with other autopsy samples negative may be a result of rather complete exsanguination by the jugular vein bleeding technic or failure to sample the organ of primary localization. The limited quantity of test material precluded satisfactory repeat isolation tests on most of the positive samples; however, reisolations were successful in 3 instances. Review of laboratory records of isolations revealed no circumstances that would have favored accidental laboratory contamination of samples. There was no correlation between the dates of introduction of newly infected birds into the aviary and the dates of autopsy of positive birds. The minimum interval was 2 months and the maximum interval was 10 months between dates of autopsy and prior introduction of newly inoculated birds.

A total of 61 tricolored and red-winged blackbirds which were uninoculated, inoculated with normal mouse brain, or inoculated with SLE virus were introduced into the aviary in the course of these studies. The SLE inoculated birds were introduced 3 months following a series of WEE birds. The birds not inoculated with virus were introduced concurrently with a WEE inoculated series. Of these 61 "control" birds, 5 converted from a negative to positive WEE neutralization index during their 4 to 7 months' residence in the aviary. These were all captured wild birds of unknown history, but previous infection with WEE virus was contraindicated by one or more serum samples which

TABLE I. History of Western Equine Encephalomyelitis Isolations from Experimentally Inoculated Birds, 1953 to 1956.

Species and date of inoculation	Sex	Days elapsed, inoc. to autopsy	WEE serum neutralization titers*			Virus isolation history†		
			Pre-inoc.	Post-inoc.	Autopsy	Tissue	Isolated in	Repeat isolations
Brewer's blackbird, 11/10/53	♂	150	0	47	Dead	Gall bladder	Chick embryo	0
Cowbird 11/10/53	♂	178	0	0	50	Lung	*Idem*	0
Idem	♂	198	0	70	32	Blood, lung	"	0
Tricolored blackbird, 2/9/54	♂	133	0	0	24	Liver	"	
Idem, 11/3/54	♂	55	0	552	352	Brain	Mouse & chick embryo	+
" , 11/29/54	♂	306	0	260	139	Spleen	*Idem*	
House finch, 11/14/55	♀	245			490	Brain	Tissue culture	+
English sparrow, 11/14/55	♂	234			0	Blood	*Idem*	+

* Serum neutralization titers against SLE were all negative.
† Other tissues routinely taken as described in the methods section were negative.

were negative on the WEE neutralization test before the final positive sample. The only blood-feeding arthropods observed in the aviary were nasal mites and the bird mite *Ornithonyssus sylviarum,* neither of which are believed to be vectors of WEE virus.

Discussion. This study demonstrates that at least one of the arthropod-borne viruses may persist in avian hosts for as long as 10 months and that virus may recirculate in the blood stream subsequent to the period of active viremia. The obvious implication of this observation is the potentiality of avian hosts with chronic latent infections to serve as long-term reservoirs and sources of virus infection for arthropod vectors. Additional studies are being directed at the evaluation of this possibility. The pattern of virus isolations from naturally infected mosquito vectors under field conditions can be interpreted as supporting the hypothesis that there is an avian source of WEE virus in the winter months(2).

Remarkably little attention has been given to the persistence of any of the arthropod-borne viruses in animal hosts beyond the period of acute viremia. The general assumption that the presence of neutralizing antibodies leads to what might be termed autosterilization of the host and particularly of the blood stream must be re-examined in the light of these findings. The recent reports of Downs(3) and Downs *et al.*(4) of the concurrent circulation of yellow fever virus and neutralizing antibodies is of interest in this regard. The current observation by Chamberlain *et al.*(5) that SLE virus persisted in the gizzard of an experimental cowbird for 38 days further supports the possible importance of birds as reservoirs as well as short-term hosts of these viruses. The earlier report of Webster and Clow(6) of SLE virus persistence for 4 to 6 weeks in the brain and spleen of resistant mice and the study of Slavin(7) in which SLE virus persisted for 162 days in the brains of passively immunized mice may be pertinent to the relationship of virus infection in resistant avian hosts.

The possible development of WEE antibodies by 5 "control" birds is the only evidence we have of bird to bird transmission in the aviary. These apparent conversions may represent errors of the neutralization test as there was insufficient serum to permit confirmatory tests.

A more detailed report of WEE and SLE latent infections in experimentally and naturally infected birds and evaluation of their ability to serve as sources of vector infection will follow completion of current studies.

Summary. Western equine encephalomyelitis virus was isolated from tissues of 8 of 284 birds at intervals of 1 to 10 months after experimental infection. Isolations were from Brewer's blackbird, cowbird, tricolored blackbird, house finch and English sparrow. Positive tissues were blood, spleen, liver, lung, brain and gall bladder. The potential importance of birds as long-term reservoirs of virus and sources of vector infection should be determined.

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Acquired Tolerance to Skin Homografts in Mice of Different Strains.* (23863)

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Actively acquired tolerance for homologous skin transplantation in mice and chickens has been achieved by exposure of these animals during fetal life to living cells from adult animals of the homologous strain(1,2). These findings were confirmed in rats(3,4). However, since inbred strains of rats were not available, tolerance to homologous skin in this species was obtained when the skin transplanted came from the donor of the spleen used to induce tolerance.

Recently Billingham and Brent(5) reported that approximately 80% of A mice injected intravenously with spleen cells no later than 24 hours after birth from an adult CBA mouse became tolerant to skin homografts taken from mice of the same stock. In contradistinction only one-half of similarly treated CBA mice became tolerant to A skin when spleen cells from the latter were used to induce this phenomenon. Moreover, when the CBA → AU donor host combination was used, not a single AU animal tolerated the CBA skin. These results would indicate the presence of strain differences in susceptibility to the induction of acquired tolerance.

It is the purpose of this report: 1. To document our confirmation of the observation of the British workers that tolerance to subsequent homotransplantation may be produced in mice by intravenous injection of spleen cells in the neonatal period. 2. To present experimental observations which indicate the existence of strain differences in capacity of the donor cells to induce tolerance as well as capacity to develop tolerance as indicated by the British workers.

Method. Highly inbred mice of the A, Ce, Z(C3H), C57 Bl(Subline 1), CBA, BALB/C and ZCe F_1 and ZBC§ hybrids were used. Acquired tolerance was induced by the method recommended by Billingham and Brent(6,7) consisting of the intravenous injection of viable spleen cells into newborn animals no later than 24 hours after birth. Cells to be in-

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§ ZBC hybrid mice are obtained in the following way:

A ♀ x Z(C3H) ♂
↓
AZ F_1 ♀ x Z(C3H) ♂ = ZBC

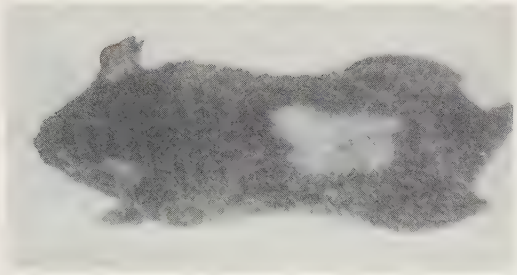


FIG. 1. Successful skin homograft in a ZBC hybrid mouse made tolerant to Ce strain.

jected were taken from the spleen of homologous adult donors. This was done by cutting one end of the spleen with a sharp scissors and gently stroking the capsule of the spleen toward the open end. In this way almost all the splenic cells were dispersed into 1 cc of Ringer-Locke's solution. The cell suspension was then passed gently through hypodermic needles varying from 26 to 30 gauge to promote further dispersion of cells. The eosin method(8) was used to determine cell viability. Each newborn mouse received from 2-4 million viable cells in 0.05 cc. The injections were made into the right anterior facial vein which runs laterally from the orbit to the right external jugular vein. Magnification of approximately 6 X facilitated the injection. Using this procedure the overall mortality due to the injection ranged from 20 to 30%. Mice surviving the injection were raised by their own mothers and weaned 25 days later. At 45 to 60 days of age animals were submitted to homologous transplantation of skin taken from adult mice isologous with the spleen donors. A piece of full thickness abdominal skin measuring approximately 1.5 x 2.0 cm was transferred to the back of the host. The graft was turned 180° and anchored with silk sutures placed at each corner and at the middle of each side of the graft. No dressings or bandages of any kind were used.

Reversing the graft facilitated determination of the subsequent success or failure of the graft since hair on the successful graft grows in a direction opposite to that on the skin of the host. Moreover, since in most host-donor combinations used the color of the skin was different, this too served as another criterion of the success of the homograft. Grafted mice were kept under observation for a period of at least 2 months.

Results. The results obtained are summarized in the table. Of the group of Ce mice injected at birth with Z(C3H) spleen cells, 3 out of 4 mice were tolerant and accepted the Z(C3H) skin homograft. In the group of ZBC hybrids treated with Ce tissue all 21 animals accepted the homologous Ce skin (Fig. 1). Of these, 3 were retested by an additional subsequent skin graft which was also successful. Both groups of ZCe F_1 hybrids injected with either C57 Bl or CBA tissue, accepted their corresponding skin homografts in all instances. Here again 4 animals among those in the later group were also tolerant to a second skin homograft. However, groups of ZCe F_1 hybrids similarly pretreated with either BALB/C or A spleen cells, uniformly failed to accept the skin homograft. These differences are significant. Attempts to homotransplant skin between adult members of the inbred strains were regularly unsuccessful as would be predicted from their genetic differences.

Discussion. These results confirm and extend those previously reported on the induction of tolerance in newborn mice injected intravenously with spleen cells of a homologous strain(1,2,5). In fact in these experiments injection of spleen cells at birth in several different donor-host strain combinations such as Z(C3H) → Ce, Ce → ZBC, C57 Bl → ZCe F_1 and CBA → ZCe F_1 produced high incidences of tolerance, while in other strain combinations, *i.e.*, BALB/C → ZCe F_1 and A → ZCe F_1 tolerance could not be in-

TABLE I. Skin Homotransplantation in Inbred Mice Pretreated by Injection of Spleen Cells at Birth.

Host strain	Donor strain	No. of mice accepting skin homografts*	No. of mice accepting 2nd skin homograft	Homograft controls
Ce	Z(C3H)	3/ 4		0/10
ZBC	Ce	21/21	3/3	0/ 7
ZCe F_1	C57 Bl	4/ 4		0/ 7
"	CBA	11/11	4/4	0/ 8
"	BALB/C	0/ 9		0/14
"	A	0/ 7		

* No. +/No. inj.

duced at all. In this regard it is interesting to point out that while ZCe F_1 hybrids became tolerant in all instances to either CBA or C57 Bl tissue, hybrids of the same kind failed to become tolerant when they are treated with either BALB/C or A tissue. Although the number of animals in these latter groups is small, the observation that the same strain of mice (ZCe F_1) may become tolerant of cells from one donor strain but not from another is of interest.

The observations of Billingham and Brent would best be interpreted as indicating differences in susceptibility to immunological tolerance on the part of the recipient strain. Our observations must be interpreted as evidence of differences in capacity of donor cells to induce tolerance upon injection in a given strain. Although apparently contradictory, these observations are not really in conflict and might indeed be based on similar underlying mechanisms.

It is clear that these striking differences in both strain susceptibility to the development of tolerance and capacity to induce tolerance were responsible for difficulties originally encountered in our laboratory when we attempted to reproduce the original observation of the British investigators.

Summary. Mice of the Ce, ZBC, and ZCe F_1 stocks have been made tolerant to homologous skin transplantation of Z(C3H), Ce, C57 Bl and CBA strains by intravenous injection at birth of homologous spleen cells. However, the same procedure failed to induce tolerance in ZCe F_1 mice when treated with homologous cells of either BALB/C or A strains.

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Improved Method for Measuring Lupus Globulin Nucleoprotein Interaction.*† (23864)

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In our recent studies a globulin factor with marked affinity for nuclei was demonstrated in lupus erythematosis serum by use of fluorescent antibody technic(1,2). Further investigation revealed that the nuclear factor in this reaction was nucleoprotein. A method for titration of the factor in lupus serum was devised. Using this test it was found that the factor was present in all serums of a large group of patients with disseminated lupus. It was also present in low titer in serum of a small proportion of patients with other syndromes generally thought to be closely allied to lupus, including especially rheumatoid arthritis, and individuals with chronic biologic false positive serologic tests for syphilis, but rarely in other human serums. In patients with positive tests there was general correlation between titer of the factor and manifestations usually associated with active disseminated lupus(3,4). These findings led to a more comprehensive clinical and laboratory study of the reaction. The possibility that some other method might be better suited to

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continued study of the reaction was investigated. In this report a method is described in which iodine^{131}-labelled antibody to human gamma globulin provided an effective means of measuring the reaction of the lupus factor with nucleoprotein.

Methods. In unpublished studies it has been shown that serums reacting with natural nucleoprotein also react with artificial complexes of DNA and histone. Natural nucleoprotein has not been sufficiently stable for use over long periods of time. Commercially available DNA and histone‡ are quite stable and the artificial complexes were therefore selected for use. DNA and histone were dissolved separately in water at concentration of 2 mg/ml. Sufficient NaCl was added to DNA solution to provide a 2 molar concentration. When this had dissolved, an equal amount of histone was added slowly with constant mixing. Salt concentration was then reduced to 0.5 molar by slowly mixing in appropriate amount of water. The final material was opalescent but contained no visible precipitated complex. Preliminary tests indicated that the most satisfactory results were obtained with glass surfaces coated with DNA-histone complex. The largest practical area was obtained by coating outside of closed end of 12x100 mm test tubes. These were cleaned thoroughly, dried and dipped in the solution of complex to depth of 6 cm. They were then suspended in horizontal position and rotated until dry. Immediately before use coated surfaces were washed by dipping quickly several times in 0.1 M NaCl to remove excess salt without dissolving the DNA-histone complex. For most studies one lot of rabbit antiserum to human gamma globulin (Fraction II) was used. This reacted at dilution of 1:256 in a ring test with 1:1000 dilution of antigen. The antiserum was fractionated with ammonium sulfate, and aliquots sufficient for several weeks work iodinated as needed by procedure described by Masouredis(5). Use of the same iodinated solution in more than one test on several occasions indicated that, under conditions used, antibody content was not a limiting factor. In the test procedure 2 ml sample of human serum was placed in tube, 16 mm outside diameter, in ice bath. The coated 12 mm tube was drained briefly, after washing as described above, and placed in serum for 10 minutes. During this period the serum was agitated several times by lifting the coated tube momentarily. At end of 10 minutes the tubes were removed from serum, rinsed gently with pH 7.0 isotonic phosphate-saline buffer and placed for 5 minutes in a larger tube containing 20-25 ml of buffer at room temperature. They were moved occasionally to facilitate washing. This step was repeated 2 more times with fresh buffer. The tubes were then placed in 2 ml of iodinated antibody solution in 16 mm tubes for 30 minutes at room temperature, then washed as before, placed in 15 x 125 mm tubes and counted in a well type scintillation counter. All tube dimensions described were based on a counter receiving a 15 mm tube. Counting of upper portion of coated area was incomplete due to instrument design, and error due to variation in coated area was therefore minimal. In one series of experiments, serums of different activities were simulated by preparing a graded series of dilutions, and tested to determine relation of recorded counts/minute to concentration of lupus factor. For most other purposes 20% concentration of serum was satisfactory. Counts/minute recorded were corrected for background radioactivity, and traces of iodine^{131} bound in absence of

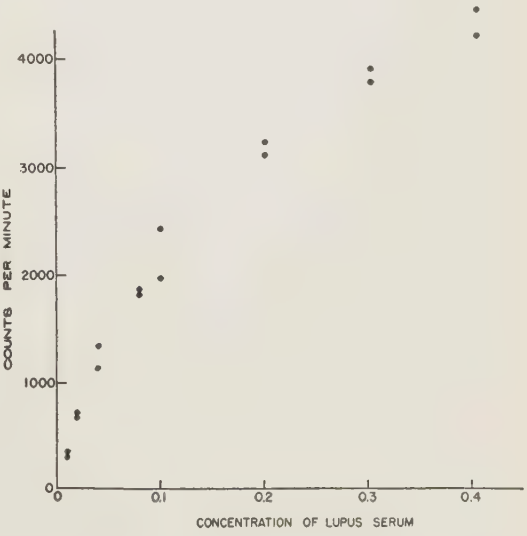


FIG. 1. Relation of concentration of lupus factor to counts/min. recorded.

‡ Worthington Biochemical Corp., Freehold, N. J.

TABLE I. Results of Tests with Iodinated Antibody as Measure of Lupus Globulin Nucleohistone Interaction.

Serum*	Conc., %	Comments	Counts/min.	
1. DLE	20		1998	2230‡
	10		1354	1546
2. "	20		584	
3. "	20	a. Control, no DNA added	4323	4215
		b. DNA added, 400 γ (μg)/ml	4084	3858
4. "	15	a. Control, no DNA or histone added	2102	2382
		b. DNA and histone added, 200 γ/ml each†	770	532
		c. DNA added, 200 γ/ml	2010	1900
5. RA	20	Low titer of affinity for	50	
6. "	20	nucleohistone with flu-	63	
7. BFP	20	orescent antibody test	49	
8. N	20	Negative against nucleo-	5	
9. "	40	histone with fluores-	3	0
10. "	40	cent antibody test	9	6
	10		0	5

* DLE, disseminated lupus erythematosis; RA, rheumatoid arthritis; BFP, chronic biologic false positive serologic test for syphilis; N, normal.

† Insoluble DNA—histone complex removed by centrifugation.

‡ Duplicate determinations. Antibody solution iodinated 17 days previously.

serum, by subtraction of results obtained in simultaneous blank determinations.

Results. In Fig. 1 results are shown which indicate relation of counts/minute to relative concentrations of the serum factor, obtained by testing a series of dilutions of a single serum specimen. Similar curves were obtained with other serums, the slope varying with activity of the particular serum. In Table I representative results of tests on selected serums are shown. In investigations of conditions which influence interaction of lupus globulin with nucleoprotein, or DNA-histone complex, controls may be included which eliminate the need for further standardization. For clinical studies the need for day-to-day standardization is apparent. Preliminary results indicate that variables can be adequately controlled for this purpose.

Our purpose has been to devise a system with which to study factors influencing the reaction of lupus globulin with nucleoprotein. Some conditions of test as now conducted have been chosen rather arbitrarily and are likely to be modified as more is learned about the reaction itself. Even in its present state of development this method appears superior to others now available.

Summary. An improved procedure has been described for measurement of interaction of globulin from lupus erythematosis serum with nucleoprotein, or artificial complexes of desoxyribonucleic acid and histone. Relatively large glass surface areas coated with these materials bound maximum amounts of the serum factor, and allowed most efficient removal of unbound serum protein by washing. Antibody for human gamma globulin labelled with iodine^{131} provided a sensitive means of measuring amount of bound globulin when used under proper conditions. Measurement of relative concentrations of serum factor was achieved in test system employing a single tube of serum at 20% concentration.

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Cultivation of Measles Virus in Stable Line of Human Amnion Cells. (23865)

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Changes which occur in primary trypsinized tissue cultures of human amnion cells infected with the Edmonston strain of measles virus are the formation of stellate-shaped cells and syncytial masses or "giant multinuclear cells" containing eosinophilic intranuclear and intracytoplasmic inclusions. Eventually, necrosis occurs and the entire culture disintegrates(1). The latter phenomenon is a slow one. Three or 4 weeks may be required for cytolysis to be complete. Occasionally the cytopathogenic changes are difficult to discern since the changes induced may be limited to the formation of 3-4 nuclei in only a few cells.

The following report describes the cultivation of measles virus in stable human amnion cells(2), and the differences in the cytopathogenic changes produced in comparison with those observed in primary human amnion cells.

Methods. Fifty thousand stable human amnion cells suspended in medium 199 containing 10% heated calf serum were inoculated into roller tubes. Within 3-4 days, the cell sheets were established and the cultures re-fed with medium 199 or Eagle medium(3), with or without glutamine, containing 10% heated calf serum. The cultures were inoculated with the Edmonston, Philadelphia 6 and Philadelphia 26(4) strains of measles virus. Infected tissue culture fluids from sheets of primary trypsinized monkey kidney epithelial (5), primary trypsinized human amnion and Hep-2 cells(6) were employed. Whole blood and mucous membrane washings from patients with measles (previously shown to contain measles virus) were used also. All tubes were rolled at 36.5°C and observed daily for evidence of cytopathogenic changes.

Results. The prominent change observed in stable human amnion in contrast to that occurring in primary amnion cultures was necrosis which became apparent 2-3 days following infection. Syncytial masses or "giant multinuclear cell" areas, though far less numerous than necrotic areas, could be seen at this time also, particularly at the periphery of the cell sheets (Fig. 1). These cytopathogenic changes were observed in all cultures inocu-

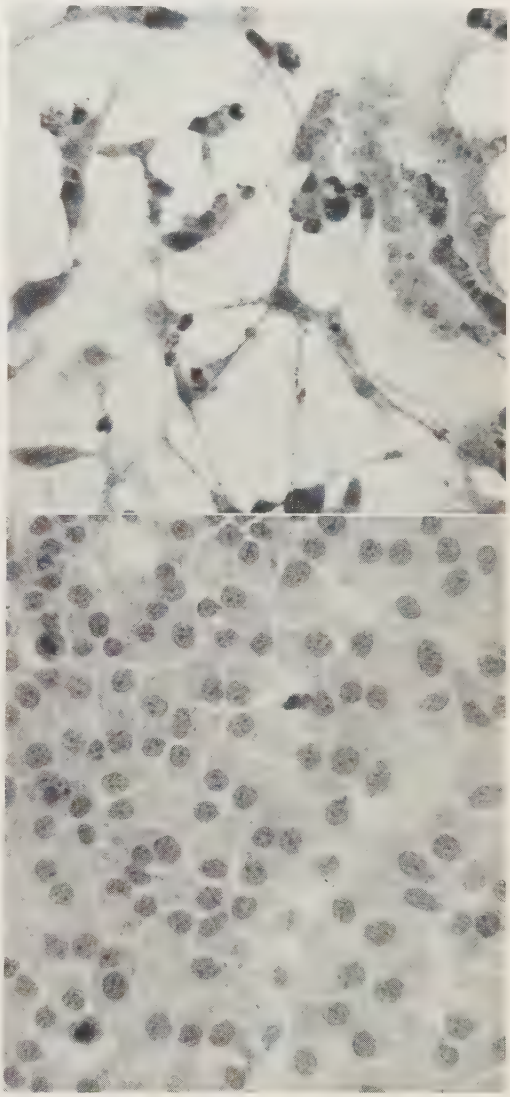


FIG. 1 (top). Stable human amnion. Culture infected with measles virus (Phila. 26 strain) 72 hr after infection. Note extensive necrosis and giant cell. Hematoxylin and eosin stain, about 150×.

FIG. 2 (bottom). Stable human amnion. Culture inoculated with mixture of measles virus (Phila. 26 strain) and measles-immune serum. One wk after inoculation. H and E stain, about 150×.

lated with measles virus, either from tissue culture fluids or clinical materials, but were not observed in tubes inoculated with mixtures of measles virus and measles-immune sera (Fig. 2). Within 7-14 days, necrosis was virtually complete even when dilute inocula were employed. Syncytial formation was almost completely suppressed by the addition of freshly prepared glutamine to the medium. The latter effect is similar to that reported in Hep-2 cultures infected with measles virus to which glutamine had been added(7).

Eosinophilic intranuclear and intracytoplasmic inclusions have been observed as early as 3 days following inoculation of large quantities of virus. Complement fixing antigen (1:8) was detected on the 4th-5th day in tissue culture fluids harvested from roller tube cultures containing 2.0 ml of medium. The average infectious titer per 0.1 ml of tissue culture fluid was 10^5 TCD_{50}. Twenty-one serial passages of measles virus have been made in stable amnion cultures.

The relative ease not only in the preparation of stable human amnion cultures but also in the early recognition of specific cytopathogenic effects suggests that these cells be used in preference to primary trypsinized human amnion cultures for measles virus studies. In our hands, these cells are preferable to either monkey kidney or Hep-2 cells in performing serum neutralization tests. They have been used also in the preparation of standard antigens for the complement-fixation test. Further work would be required to determine their general usefulness in the isolation of measles from clinical materials.

Summary. Cytopathogenic changes induced by measles virus propagated in a stable line of human amnion cells is described. The prominent change is necrosis in contrast to formation of "giant multinuclear cells" occurring in primary human amnion cultures infected with measles virus. Preliminary studies indicate the general utility of this line of cells for investigations on measles virus.

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Effect of Reserpine on Prolactin Content of Rabbit Pituitary.* (23866)

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A single intravenous injection of reserpine into mature female rabbits induced a limited degree of lactation after prior treatment with estrogens or at the end of pseudopregnancy (1-3). Administration of reserpine(3) and chlorpromazine(4-7) have also been reported to occasionally initiate lactation in women patients. These results, as well as the finding that reserpine may prolong luteal function in the rat(8), suggests that this drug may stimulate production and release of prolactin from the pituitary. The present experiment demonstrates that reserpine can increase the prolactin content of the pituitary of rabbits.

Methods and results. Twenty-two mature female rabbits, predominantly New Zealand White in origin, were divided into 4 groups of 5 to 6 each. The rabbits averaged 6-9 pounds in body weight and were fed a standard rabbit diet. Treatment of the 4 groups was as follows: 1, controls; 2, a single intravenous

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† Reserpine was kindly provided by Dr. Robert Gaunt of Ciba Pharmaceutical Products, and by Dr. Russel Kraay of Eli Lilly and Co.

TABLE I. Effects of Reserpine and/or Estradiol on Prolactin Content of Pituitary.

Group & total No. of rabbits	Treatment	Avg pit. wt, mg	Avg pigeon units/pit.	Avg pigeon units/mg pit.
(1) 5	Controls	30.4	0.37 (.016)§	.012 (.0005)§
(2) 5	Reserpine*	31.7	2.00 (.090)	.060 (.0027)
(3) 6	Estradiol†	38.4	4.75 (.214)	.123 (.0055)
(4) 6	Estradiol & reserpine‡	38.9	6.75 (.304)	.170 (.0076)

* Reserpine inj. once and rabbits killed 1 wk later.
† Estradiol inj. for 10 days and rabbits killed 1 wk after last inj.
‡ Estradiol inj. for 10 days followed by 1 inj. of reserpine, and rabbits killed 1 wk later.
§ International Units [1 I.U. = 22.2 pigeon units(9)].

injection of 1 mg reserpine/kilo body weight and killed 1 week later; 3, 0.2 mg estradiol daily for 10 days and killed 1 week later; 4, 0.2 mg estradiol daily for 10 days, followed by a single intravenous injection of 1 mg reserpine/kilo body weight on the 10th day, and killed 1 week later. The pituitary from each rabbit was removed, weighed to the nearest 0.1 mg and combined from each group for assay. They were macerated into a paste with a small agate mortar and pestle, taken up as a fine suspension in distilled water, and assayed in mature White Carneau pigeons by the sensitive intradermal technic(9). The 5 pituitaries from the control rabbits were directly compared in the same 10 pigeons against the 5 pituitaries from the reserpine-injected rabbits by injecting over both sides of each crop sac. Since estrogens increase pituitary prolactin content(10), the equivalent of only 2 pituitaries from the estradiol-injected rabbits was directly compared in 10 pigeons against the equivalent of 2 pituitaries from the rabbits given both estradiol and reserpine. Representative mammary glands from each of the rabbits were examined for gross and microscopic evidence of growth and lactation.

Results. A single injection of reserpine (group 1) increased pituitary prolactin content at least 5-fold as compared to the controls, but did not initiate lactation (Table I). There was no evidence that reserpine induced a significant degree of mammary growth. Injections of estradiol (group 2) increased the prolactin content of the pituitary at least 10-fold as compared to the controls. Although some growth of the mammary duct system occurred in these animals, milk secretion was not initiated. The greatest increase in pituitary prolactin content was elicited when estradiol was followed by reserpine (group 4). The mammary glands of all these rabbits showed considerable lobule-alveolar development and a limited but definite degree of lactation. This was described previously(1). In most of the rabbits given reserpine, drowsiness or sleep was induced for several hours after injection of the drug.

Discussion. These results show that a single intravenous injection of a relatively large dose of reserpine elicits a pronounced increase in the prolactin content of the pituitary of mature female rabbits. A single injection of reserpine alone, however, failed to initiate lactation, presumably because the mammary glands remained rudimentary in structure. Treatment with estradiol alone for 10 days produced a greater increase in pituitary prolactin than reserpine, but mammary growth was only slightly stimulated and lactation was not initiated. Long-term treatment of rabbits with estrogens has induced extensive mammary growth, increases in pituitary prolactin content and lactation(11,12). When reserpine was given following injections of estradiol for 10 days, there was considerable lobule-alveolar development, an increase in pituitary prolactin and milk secretion. This suggests that the presence of adequate mammary tissue is essential for the prolactational action of reserpine in the rabbit.

In this laboratory, injections of reserpine or chlorpromazine failed to induce lactation when injected into mature female rats for 20 days, and did not increase milk production when injected into parturient lactating rats or into goats during the declining phase of lactation (unpublished results). Other investigators have independently observed that these drugs do not induce lactation in rats or guinea pigs, nor increase milk yields in goats(3,13). We have injected both drugs into mature

White Carneau pigeons for 20 days without eliciting crop milk secretion. It appears therefore, that a species difference exists in the ability of these drugs to influence prolactin secretion and perhaps other hormones which regulate mammary growth and lactation.

Summary. 1. A single intravenous injection of 1 mg reserpine/kilo/body weight into 5 mature female rabbits, followed by sacrifice 1 week later, resulted in at least a 5-fold increase in pituitary prolactin content over 5 controls. There was no evidence that reserpine induced mammary growth or lactation. 2. Subcutaneous injections of 0.2 mg estradiol daily for 10 days into 6 rabbits elicited 1 week later at least a 10-fold increase in pituitary prolactin content, a slight growth of the mammary duct system and no milk secretion. 3. When estradiol treatment was followed by a single intravenous injection of reserpine (above dose) and the rabbits killed 1 week later, pituitary prolactin content was at least 16 times greater than in controls. This was accompanied by a considerable degree of lobule-alveolar development and a limited amount of milk secretion in all 6 animals.

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Potentiation of Barbiturate Hypnosis by Certain Uncoupling Agents.*†‡ (23867)

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The hypothesis that barbiturates produce their depressant effects by uncoupling oxidative phosphorylation has been proposed(1-4). An objection to this premise is the observation that other uncoupling agents are not hypnotics or anesthetics in the intact animal. The possibility that many uncoupling agents fail to depress *in vivo* because of limited penetrability into the central nervous system has been discussed(4). While most uncoupling agents possess little or no hypnotic properties *per se,* the present study will demonstrate that they potentiate hypnosis induced by the barbiturates. The uncoupling agents studied were: 2,4-dinitrophenol, (D NP) 2,4-dichlorophenoxyacetic acid (2,4-D) and chlorotetracycline (Aureomycin).

Methods. Sleeping time was measured in white mice of the Harlan strain of both sexes weighing 18 to 25 g. The test drugs or saline solutions were made up in such a manner as

* This study was initiated at Department of Pharmacology, University of Illinois, Chicago, and a preliminary report presented at Fall Meeting of Am. Soc. for Pharmacol. and Exp. Therap., Madison, Wisc., 1952.

† With the technical assistance of Ruth Hurwitz and Mary Alice Ratchford.

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to administer the same volume/unit weight to all animals. All test drugs or saline were administered to mice 30 minutes prior to the challenging dose of the barbiturate with the exception of the adrenergic blocking agents which were administered 1 hour prior to the barbiturate. The "sleeping time" was designated as the interval between the loss and return of the righting reflex, or more specifically, the "on side time." To minimize variations in sleeping time from external stimuli, a regularly intermittent jet of air was played over all animals. The sequence of injections for each treatment time was randomized. In each experiment, injections were made by one person and observations performed and recorded by another. Separate additional control groups were sometimes run at the end of an experiment (Table I). The statistical significance of the differences between various groups was analyzed by the conventional "t" test in Tables I and II. In the case of Table III the experimental design was arranged so that the results could be statistically evaluated by the use of factorial coefficients in the analysis of variance. Specifically, equal numbers of animals were used in all treatment groups, and each experiment contained representative cells from each treatment group. Sprague-Dawley rats were used in studies designed to determine whether or not the uncoupling agents interfered with mechanisms for barbiturate degradation. The animals in this study were pretreated with 20 mg/kg I.P. of DNP or saline in equal volume/unit weight. Forty minutes after pretreatment, 40 mg/kg of secobarbital was administered intravenously. Blood samples were obtained from tail vein at 5, 10, 20, 40 and 60 minutes after administration of barbiturate and analyzed. Blood secobarbital levels were determined by a modification of the method of Brodie *et al.*(5). Similar experiments were carried out in mice except in this instance serial samples from a single animal could not be used because of blood volume required for analysis. Consequently, groups of animals were sacrificed at each time point and comparison between groups was made rather than utilizing a given animal as its own control. Mice were given various doses of DNP and

TABLE I. Potentiation of the Hypnotic Effect of Barbiturates by Certain Uncoupling Agents.*

No. animals	Uncoupling agent	Dose, mg/kg	Sleeping time, min.†	"P"‡
Exp. 1				
20	None		24.4 ± 3.3	
20	Dinitrophenol	20	46.0 ± 2.6	<.001
20	Chlorotetracycline	50	38.8 ± 4.7	<.05
Exp. 2				
25	None		25.0 ± 3.4	
25	Dinitrophenol	20	45.8 ± 2.2	<.001
25	Chlorotetracycline	50	43.2 ± 5.6	"
25	2,4-dichlorophenoxyacetate	50	63.9 ± 5.1	"
10	None	50	23.8 ± 1.6	n.s.
Exp. 3				
16	None		31.6 ± 3.8	
8	Dinitrophenol	20	51.2 ±10.6	<.05
8	Chlorotetracycline	25	28.4 ± 5.6	n.s.
8	"	50	73.4 ±13.7	<.01
16	2,4-dichlorophenoxyacetate	50	71.3 ± 6.0	<.001
8	None		49.3 ± 7.4	<.05
Exp. 4				
10	None		0 (ataxia only)	
20	Dinitrophenol	20	5.2 ± .44	

* All animals were given a challenging dose of 50 mg/kg secobarbital (amobarbital in Exp. 4) 30 min. after pretreatment with the indicated uncoupling agent as described under Methods.

† Expressed as mean ± stand. error.

‡ 1st set of barbiturate controls *vs* the indicated group.

sacrificed by decapitation at various times as indicated in Table IV. The brains were rapidly removed, weighed, and homogenized in water. Blood was collected in heparinized tubes and centrifuged at 8000 x g for plasma recovery. Suitable aliquots of homogenate and plasma were taken for DNP estimation using the analytical method of Mudge and Taggart(6).

Results. Under the experimental conditions described and the dosages employed, all uncoupling agents used, 2,4-dinitrophenol (DNP), chlorotetracycline and 2,4-dichlorophenoxy acetic acid, (2,4-D) significantly increased sleeping time of mice treated with secobarbital. Sleep also was observed in animals which were pretreated with DNP and subsequently challenged with a dose of 50 mg/kg of amobarbital injected intravenously. This dose of amobarbital produced only a slight ataxia in a group of mice pretreated with isotonic saline. These data are shown

TABLE II. Effect of Various Doses of DNP on Hypnotic Effect of Secobarbital,* 11 Animals per Series.

DNP, mg/kg	Sleeping time, min. (mean ± S.E.)	"P"†
None	13.0 ± 2.33	
5	29.0 ± 5.67	<.01
10	35.0 ± 5.71	<.001
15	36.0 ± 5.71	"
20	49.0 ± 5.02	"
None	19.0 ± 3.12	

* All animals were given a challenging dose of 40 mg/kg secobarbital 30 min. after pretreatment with the indicated dose of DNP as described under Methods.

† Combined secobarbital control groups *vs* secobarbital + DNP.

in Table I. In Exp. 3 the control group run at end of the day differed significantly from control group run earlier. Also this control group did not differ significantly from the DNP treated group although it did differ significantly from the chlorotetracycline and 2,4-D treated groups. Since this happened on only one occasion we have not given this result much weight. Pretreatment with DNP at 5, 10, 15 and 20 mg/kg and subsequent administration of a standard dose of secobarbital progressively increased duration of sleeping time (Table II). At the higher doses of DNP, some animals were hyperthermic, and a slight depression of normal activity could be observed prior to administration of the barbiturate.

Pretreatment of animals with the adrenergic blocking agents, dibenamine and yohimbine, failed to antagonize the increase in sleeping produced by the uncoupling agent. The adrenergic blocking agents *per se* significantly prolonged the duration of sleeping time when compared with saline-pretreated mice. In combination with DNP both dibenamine and yohimbine were apparently additive in enhancing these depressant effects (Table III).

Side effects noted with administration of the blocking agents were hyperactivity with yohimbine and slight depression with dibenamine. In combination with DNP, neither agent modified the hyperthermia produced by the administration of DNP. The gross effects on motor activity produced by the blocking agents were unchanged by the administration of DNP.

With both rats and mice no significant difference was noted in the disappearance of barbiturate from the blood of control animals and animals previously treated with DNP. The variability was quite high in mouse experiments, but in rat experiments where each animal was his own control more consistent results were obtained.

Measurable amounts of DNP reach the brain, maximum concentration observed 30 minutes after i.p. administration (Table IV). Since we did not obtain very good recoveries (perhaps because we did not use mechanical shaking for extractions) the values given in Table IV must be minimal. In animals sacrificed by cardiac puncture the blood content of brain was approximately 0.02 ml/g(7). Thus 5.8 of the 17.9 millimicromoles DNP observed in brain at a 20 mg/kg dose might be from the blood content. However, correction for recovery would just about balance correction for blood content and we conclude that the brain concentration would be about 1.5×10^{-5} M at 20 mg/kg.§

Discussion. It has been shown that bar-

TABLE III. Combined Hypnotic Effect of DNP, Adrenergic Blocking Agents and Secobarbital.

No. animals	Adrenergic blocking agent, mg/kg I.P.	DNP, mg/kg	Seco-barbital, mg/kg	Sleeping time, min., mean ± S.E.
	Dibenamine			
27	50	None	40	30.5 ± 9.6
27	50	15	"	39.8 ±12.7
27	None	None	"	17.9 ± 5.4
	Yohimbine			
27	15	None	40	26.0 ± 7.9
27	15	15	"	37.8 ± 6.8
27	None	15	"	28.7 ± 9.1

DNP *vs* no DNP, "P" = <.001.

Adrenergic blocking agent *vs* no adrenergic blocking agent, "P" = <.001.

Yohimbine *vs* dibenamine—not significant.

§ Goldman, Laatsch, Molison, Urata and Hunter at Washington University in St. Louis observed blood levels of about 80 millimicromoles/ml and brain levels of about 3.5 millimicromoles/g in rats after 40 mg/kg i.p. doses (personal communication). We have no explanation for the difference between our results and theirs.

TABLE IV. 2,4-Dinitrophenol in Mouse Brain and Plasma (Effect of Dose and Time).

Values expressed as millimicromoles/g tissue or/ml plasma and are averages of determinations on No. of animals given in parentheses.

Avg recovery from tissue in 10 experiments was 65% (range: 51-86%); from plasma in 3 experiments was 80%. Tabular values are not corrected.

	Dose†			
	10 mg/kg	20 mg/kg	40 mg/kg	60 mg/kg
Brain	7.0*(4)	17.9(8)	56.4(8)	128.6(2)
Plasma	139 (2)	290 (6)	425 (8)	822 (2)

	Time‡			
	15′	30′	45′	60′
Brain	47.7 (2)	51.3(2)	32.6(2)	28.0(2)
Plasma	518 (2)	440 (2)	417 (2)	272 (2)

* Method will detect approx. 8 mμmoles; tissue and plasma blanks are essentially zero. However, the 10 mg/kg tissue value is not reliable.

† Animals were given the doses of NaDNP indicated intraper. 30 min. prior to sacrifice by decapitation.

‡ Dose, 40 mg/kg.

biturates uncouple oxidative phosphorylation *in vitro,* and it has been postulated that this uncoupling is a basis of the central nervous system depressant action of the barbiturates. 2,4-dinitrophenol also uncouples oxidative phosphorylation *in vitro,* but when given alone *in vivo* shows very little depressant activity. Only a small portion of a given dose of DNP reaches the central nervous system. The concentration observed at 20 mg/kg (1.5×10^{-5} M) is sufficient to produce a pronounced uncoupling effect *in vitro*(3). The effectiveness of this concentration on metabolic processes *in vivo* is a matter for conjecture. However, if an animal is under the influence of a barbiturate, then the small amount of DNP which does reach the brain may exert a noticeable depressant effect by the addition of the uncoupling effects of these two agents. This explanation could account for the present findings. DNP is a somewhat more potent uncoupling agent *in vitro* than the other two uncoupling agents hence we used somewhat higher doses of chlorotetracycline and 2,4-D in the present experiments. Our data are not sufficiently extensive, however, to permit any correlation of potentiation potency *in vivo* with uncoupling potency *in vitro.*

A large number of compounds has been shown to potentiate barbiturate anesthesia. These include: glucose(8); potassium ion (9); epinephrine, lactate, glutamate and other carbohydrate intermediates(10,11); SKF 525 A(12); dimercaprol(13); antabuse(14); and isoniazid(15). The mechanisms by which the above compounds potentiate barbiturate anesthesia do not include the uncoupling of oxidative phosphorylation. The potentiation of barbiturate anesthesia by the carbohydrate intermediates may be mediated by epinephrine. Dimercaprol, SKF 525 A and isoniazid apparently interfere with systems concerned with barbiturate detoxication, while potentiation with antabuse can be demonstrated for many depressants unrelated to the barbiturates(16). We have attempted to rule out the possibility that the uncoupling agents are potentiating anesthesia *via* an epinephrine release by use of adrenergic blocking agents. The latter compounds failed to prevent the potentiating effects of the uncoupling agents and actually increased barbiturate sleeping time, an interesting finding in itself. Our studies on blood barbiturate levels after DNP would also seem to negate the possibility that an inhibition of barbiturate degradation is involved. Chlorpromazine, a compound which will also potentiate barbiturate anesthesia, has been reported to uncouple oxidative phosphorylation(17), although the potentiation observed with this compound might obviously be attributable to its adrenergic blocking properties.

The authors recognize that explanations other than addition of uncoupling effects are possible to account for the observed potentiation. We have attempted to eliminate effects of detoxification and epinephrine release. Studies on possible potentiation of the anesthetic effects of classes of drugs other than the barbiturates and the use of other uncoupling agents may yield data germane to the general problem. We are further investigating metabolic changes brought about by these drugs both singly and in combination to obtain direct data bearing on the question of uncoupling *in vivo.*

Summary. It has been demonstrated that certain uncoupling agents increase the hypnotic effects of certain barbiturates. Potentiation by uncoupling agents is not the result

of inhibition of detoxication mechanisms nor is epinephrine release implicated since the effect is not prevented by adrenergic blocking agents. The nature of this effect and its relationship to the mechanism of action of the barbiturates is discussed.

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Turnover of 5-Hydroxytryptamine (Serotonin) in Tissues.* (23868)

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Development of sensitive and specific analytical methods for serotonin (5-hydroxytryptamine)(1,2) has made it possible to demonstrate its presence in many tissues. Thus, although the bulk of serotonin is found in mucosa of gastro-intestinal tract, significant amounts are also found in blood platelets, brain, spleen and lung. Enzymes involved in serotonin formation and destruction are present in all depots, except blood platelets, indicating that the amine is apparently made, released and destroyed for specific functions in these depots. It is hoped that these studies on turnover of serotonin in various tissues may be helpful in elucidating the nature of these functions.

Materials and methods. A solution of serotonin hydrochloride was prepared in the following manner. Serotonin creatinine sulfate was dissolved in water adjusted to pH 10 and the solution saturated with NaCl. Serotonin was extracted into n-butanol, and after addition of heptane it was reextracted into dilute HCl. The serotonin concentration was standardized colorimetrically. *DL*-tryptophan 2-C^{14} (specific activity 0.3 $\mu c/\mu M$) was obtained from Tracerlab Inc. Radioactive 5-hydroxytryptophan (5HTP) was synthesized in this laboratory following procedure outlined by Ek and Witkop(3). The amino acid was labelled in the β-carbon of the side chain and had a specific activity of 0.1 $\mu c/\mu M$. Radioactivity measurements were made with Robinson gas flow proportional counter having background of 2 counts/minute. Counts were corrected to constant weight, based on experimentally determined self-absorption curve, and expressed as counts/minute/μmole of compound. Serotonin in tissues was isolated by previously

* The authors wish to express their thanks to Mrs. Doris Titus for carrying out synthesis of 5HTP-C^{14} and to Mrs. Betty G. Redfield for studies with harmaline.

TABLE I. Labelling of Tissue Serotonin following Tryptophan-C^{14}.

Days following last inj. of tryptophan-C^{14}	Tissue	Serotonin, cpm/μMole
4	Platelets	1239
9	"	285
9	Spleen	264
9	Stomach	103
9	Intestine	97

described procedure employing butanol extraction followed by re-extraction with dilute acid(1). One aliquot of final acid extract was assayed for serotonin spectrophotofluorometrically(4) and 10 mg of serotonin hydrochloride were added to the remainder of extract. The extract, containing carrier, was evaporated under nitrogen to 0.2-0.3 ml and the amine was precipitated by dropwise addition of a saturated aqueous solution of picric acid. The picrate salt was then recrystallized to constant specific activity. Results obtained by this procedure were frequently checked by procedure employing chromatographic isolation on Whatman #1 paper (propanol-1 N NH_3, 5:1) of the carrier-free serotonin in the final acid extract. The 2 methods were always in excellent agreement. Radioactivity studies were carried out on adult rabbits. Tryptophan-C^{14} was administered intraperitoneally every day for 7 days, 2.7 mg, containing 4 μc/day. 5HTP-C^{14} was given in single intraperitoneal doses of 9.6 mg. Harmaline† was administered intraperitoneally to rats in doses of 20 mg/kg and the studies of effect on brain serotonin levels were carried out as described previously (5). Blood platelets were isolated according to procedure of Dillard *et al.*(6).

Results. Following administration of its precursors in radioactive form the various body pools of serotonin became labelled. Disappearance of radioactivity from serotonin in these depots can be taken as a measure of turnover of this amine in the particular tissue. Both tryptophan-C^{14} and 5-hydroxytryptophan-C^{14} were administered to rabbits for such studies. The data obtained in various tissues are summarized in Table I and Fig. 1.

† Harmaline (free base) was generously supplied by Pfizer and Co.

Platelets. Data on platelet serotonin turnover are summarized in Table II. Following administration of tryptophan-C^{14}, 3-4 days were permitted to elapse before drawing first blood sample. By this time the specific activity of free plasma tryptophan was much lower than that of platelet serotonin. Following 5HTP, the amino acid persisted for several hours in the tissues. The initial level for platelet serotonin was taken 5 hours after 5HTP injection. In both cases the fall in specific activity of platelet serotonin can be taken as index of turnover of serotonin in this tissue. Experiments with tryptophan-C^{14} and 5HTP-C^{14} yielded half-lives of 48 and 33 hours respectively. A half-life of this order of magnitude is not inconsistent with reported values of platelet survival time(7,8). Studies with blood platelets indicate that this tissue contains little, if any catalysts involved in serotonin formation or destruction and that the amine is taken up by the platelet from surrounding fluids(9-11). The resulting binding by the platelet is such that serotonin appears to be liberated mainly as a result of platelet breakdown.

Spleen. It is apparent from data in Fig. 1 that the turnover of serotonin in spleen parallels that of platelet serotonin. Nine days after administering tryptophan-C^{14} similar specific activities were also obtained for platelet and spleen serotonin (Table I). It would appear that the bulk of serotonin in spleen may be there in platelets stored in this organ.

Gastro-intestinal tract. The mucosa of gastro-intestinal tract comprises the major de-

TABLE II. Turnover of Serotonin in Platelets.

	Tryptophan-C^{14}		5HTP-C^{14}	
Time	cpm/μM	Fraction remaining	cpm/μM	Fraction remaining
Initial	1239	1.00	32,800	1.00
24			19,350	.59
48	900	.73	14,800	.45
72			7,960	.24
96	390	.31		
120	285	.23		
Calculated half-life	48 hr		33 hr	

Each value represents avg obtained on 2 animals from which blood samples were obtained at times indicated. Animals used in tryptophan-C^{14} studies weighed 2.5–3.0 kg. Those used for 5HTP-C^{14} studies weighed 1.5–2.0 kg.

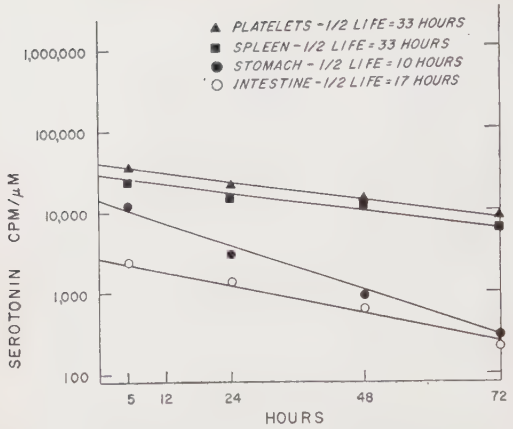


FIG. 1. Half-lives of serotonin in rabbit tissues following administration of 5HTP-C^{14}.

pot of serotonin. It is apparent from data in Fig. 1 that serotonin in these tissues turns over more rapidly than does platelet serotonin. That serotonin in stomach and intestine have similar turnovers is further suggested by data in Table I. Stomach and intestine, in the rabbit, contain approximately 10-20 μg of serotonin/g of tissue. Half lives of 10-17 hours indicate that large quantities of this substance are released daily from these tissues. Release of such quantities of serotonin is consistent with suggestions that serotonin has a regulatory function in the gastro-intestinal tract(12) perhaps as a regulator of peristalsis(13).

In contrast to the rapid turnover of serotonin in rabbit gastro-intestinal mucosa are the findings of a fairly slow serotonin turnover (half-life about 5 days) in malignant carcinoid tumors in man(14). Since serotonin in both gastro-intestinal mucosa, and carcinoid tumors is associated with enterochromaffin tissue, the difference in serotonin turnover is surprising. It will be important to determine whether this represents a species difference or a difference between normal and neoplastic tissue.

Brain. Several attempts were made to determine half-life of brain serotonin using 5HTP-C^{14}. However, it was apparent that entrapment of traces of blood, containing approximately 4 μg/ml of serotonin with a relatively long half-life, would seriously influence brain serotonin, 0.5 μg/g. Although values of less than 10 hours were obtained by the isotopic method, they were not considered to be even an index of maximal half-life values.

In a previous report it had been shown that following administration of the monoamine oxidase inhibitor, isopropyl-isonicotinyl hydrazine (marsilid) there was a rapid rise of brain serotonin. In both rabbits and rats marsilid produced large increases in brain serotonin, the serotonin content doubling in 2-3 hours (5). This rapid increase following marsilid suggested a rapid rate of serotonin turnover in brain. It was considered that the turnover was even faster than was apparent from the rise in serotonin since as *in vitro*, marsilid may require some time to become an effective inhibitor of serotonin metabolism. More recently it has been possible to find more potent inhibitors of monoamine oxidase(15,16). An analogue of one of these, harmaline, has been studied as a monoamine oxidase inhibitor both *in vitro* and *in vivo* (to be published). When harmaline was administered to rats there occurred an extremely rapid increase in brain serotonin levels. A typical experiment, Table III, indicates that doubling of brain serotonin occurs within minutes. This is much more rapid than was obtained with marsilid. If this is now taken as the index of serotonin turnover in brain then it would certainly suggest rapid formation and release of this amine in the central nervous system.

Other tissues. Serotonin is also found in the lung of many animal species and in skin of rats and mice. However, these studies were carried out before serotonin localization in these tissues was known.

Discussion. It is obvious that the various

TABLE III. Increase in Brain Serotonin following Administration of Harmaline.

Time, min.	Brain serotonin, μg/g
0 (controls)	.40
10	.60
20	.73
30	.80
60	.87

Harmaline, 5 mg/kg, was administered intraper. to adult rats following which the animals were sacrificed at indicated intervals. Each value comprises pooled brain from at least 3 animals, except for controls which are pooled averages of 9 animals.

depots of serotonin differ from one another in many respects. Although total amount of serotonin in brain is small compared to that in the gastro-intestinal tract it is apparent that synthesis and metabolism of serotonin occurs much more rapidly in the central nervous system than it does peripherally. This may also be true for norepinephrine since levels in the brain also rise rapidly following administration of monoamine oxidase inhibitors(15). However, with catecholamines too, peripheral depots, such as those in adrenal gland are known to turn over very slowly(17). The rapid turnover of these amines in the central nervous system is indicative of an important function.

Serotonin in blood platelets is quite distinct from that in other tissues. Its turnover does not reflect synthesis and metabolism by platelets. The amounts of serotonin in this tissue depend on amounts released from other depots most probably the gastro-intestinal tract. This would explain increased platelet levels in malignant carcinoid. The turnover in platelets is, therefore, a function of uptake and platelet survival.

Summary. Using tryptophan-C^{14} and 5-hydroxytryptophan-C^{14} it has been possible to measure turnover of serotonin in a number of tissues in the rabbit. The measured half lives are: platelets and spleen, 33-48 hours; stomach, 17 hours; intestine, 11 hours. Although amounts of serotonin in brain were too small to permit isotopic measurement it was possible to estimate its turnover from the increase following administration of harmaline, an inhibitor of serotonin metabolism. Such estimates indicate a half-life in brain of the order of minutes.

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Effect of N-Acetyl-para-Aminophenol on Plasma Levels of 17-Hydroxycorticosteroids.* (23869)

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Hench(1) observed that rheumatoid arthritis patients experienced marked or complete temporary remissions of arthritis during bouts of spontaneous intercurrent jaundice. Although there was some correlation between extent of remission and serum bilirubin concentration, Hench was of the opinion that bilirubin was not responsible for remission of

* Presented in part before the Canadian Physiol. Soc., Ottawa, Oct. 1957.

arthritis. It is now known that urinary bilirubin, in jaundiced patients with bilirubinuria, is present almost exclusively in the form of glucuronide(2), as are urinary adrenocorticoids to a large extent(3,4). It is conceivable that remissions of arthritis observed by Hench (1) may have been due, at least in part, to elevated adrenocorticoid levels arising from competitive inhibition by bilirubin of steroid-glucuronide formation in the liver. The elevated plasma levels of 17-hydroxycorticosteroids observed in patients receiving large doses of diethylstilbestrol(5) and of salicylates(6) may possibly be explained in terms of a similar inhibition since these substances also are glucuronide precursors. Thus, substances which readily undergo conjugation with glucuronic acid may inhibit steroid conjugation *in vivo* by competing with steroid for available glucuronide-forming enzyme system. Should this be so, one would expect to find, upon administration of a steroid along with non-steroid glucuronide precursor, an elevated free steroid plasma level. N-Acetyl-para-Aminophenol (NAPA), when administered to humans, appears in urine in conjugated form to 85-96%, mainly as glucuronide and partly as ethereal sulfate(7,8). Single 2 g doses, administered orally to humans, caused 200% increase in urinary glucuronide output(9). In rabbits, it was more than 3 times as effective as acetylsalicylic acid as regards its ability to stimulate output of urinary glucuronide (9). The present paper is concerned with the effect of NAPA on plasma levels of free 17-hydroxycorticosteroids (17-OHCS) arising from exogenous hydrocortisone (cortisol) and prednisolone (Δ^1-cortisol) administered to rabbits.

Materials and methods. Female albino rabbits weighing 3 to 4 kg were used. Plasma levels of free 17-hydroxycorticosteroids (17-OHCS) were determined 1 or 2 hours after a single dose of cortisol or prednisolone, as indicated in the Tables. After 72 hour interval the steroid and NAPA were administered concurrently and plasma 17-OHCS again measured. (24 hours after administration of a single dose of cortisol or prednisolone there was no detectable 17-OHCS in plasma and hence no carry-over from one experiment to the next.) NAPA was administered by mouth as solution or suspension in tap water. Cortisol and prednisolone were dissolved in 50% propylene glycol, and injected intraperitoneally 30 minutes after NAPA had been given. Blood samples were obtained by cardiac puncture into heparinized syringes. Plasma was separated by centrifugation, and samples were stored in deep freeze until used for 17-OHCS determination. Plasma 17-OHCS were determined by the Silber-Porter method(10), as modified by Peterson *et al.*(11), 2 ml of plasma being used generally for each determination. Circulating 17-OHCS bearing a free Δ^4-3-oxo group were determined by the INH reaction of Umberger(12); and total glucuronic acid by the method of Fishman *et al.*(13), with the exception that pigment produced in the color reaction was extracted with n-butyl acetate in place of toluene.

Results. NAPA (250 mg/kg body weight), administered concurrently with cortisol (25 mg/kg), produced a marked increase in free 17-OHCS plasma levels, as measured by Porter-Silber method (Table I). At 1 and 2 hour intervals after dosage, the mean plasma level of 17-OHCS, in the presence of NAPA

TABLE I. Effect of N-Acetyl-p-Aminophenol (NAPA) on 17-OHCS* Plasma Levels in Rabbits, following Administration of 25 mg/kg of Hydrocortisone, Intraperitoneally.

Hr after dosage	17-OHCS plasma levels: Without NAPA, μg/ml	With NAPA,† μg/ml	% of control
1	5.25	9.30	177
1	2.20	4.20	191
1	3.90	10.20	262
1	6.40	10.10	158
1	4.40	9.40	214
1	2.30	9.70	422
1	3.20	7.10	222
‡	3.95 ± .24	8.56 ± .87	235 ± 34
2	4.97	11.15	224
2	5.81	13.13	229
2	4.68	17.62	376
2	5.44	13.87	255
2	6.94	12.23	176
2	5.42	13.89	256
‡	5.54 ± .32	13.68 ± .9	253 ± 27.3

* Determined by Peterson modification(10) of Porter-Silber method(9).

† N-Acetyl-p-Aminophenol (Atasol, Horner), 250 mg/kg body wt administered orally 30 min. before hydrocortisone.

‡ Mean ± S.E.

was 235 ± 34 and 253 ± 27%, respectively, of that attained when cortisol alone was given. A similar increase was obtained at cortisol dosage of 10 or 50 mg/kg and NAPA at 250 mg/kg.

No detectable amount of 17-OHCS was found in plasma of untreated rabbits or those administered an oral dose of NAPA alone. These results are in agreement with those of Done *et al.*(14), who found no circulating 17-OHCS in rabbit plasma even after ACTH stimulation. In this connection, Bush(15) found corticosterone to be the major adrenal steroid present in rabbit plasma. It is probable, therefore, that the 17-OHCS present in plasma of cortisol-treated rabbits was contributed entirely by exogenous cortisol.

It may be supposed that increased plasma levels of Porter-Silber reactive materials may consist predominantly of tetrahydro derivatives of cortisol rather than Δ^4-3-ketocorticosteroids. To obtain information on this point the isoniazid (INH) reaction of Umberger(12) was utilized. This reaction involves the formation of isonicotinylhydrazones of Δ^4-3-ketocorticosteroids, which can be measured colorimetrically, and the reaction has been shown to be specific for the Δ^4-3-keto group. The amounts of free Porter-Silber and INH-reactive materials were determined in aliquots of plasma from rabbits treated with cortisol alone and with cortisol plus NAPA. In each case the values for INH-reactive materials were essentially the same as those obtained with the Porter-Silber method, or only slightly lower. These results (Table II), indicate that increased plasma levels of 17-OHCS obtained after concurrent administration of cortisol and NAPA are comprised mainly of biologically active steroids, or steroids which are potentially so, since they contain the Δ^4-3-keto group.

TABLE II. Comparison between Porter-Silber and INH-Reactive 17-OHCS in Plasma, following Administration of 25 mg/kg Hydrocortisone Intraperitoneally.

Porter-Silber (17-OHCS), μg/ml		INH (Δ^4-3-KCS), μg/ml	
Without NAPA	With NAPA, 250 mg/kg	Without NAPA	With NAPA, 250 mg/kg
1.64	3.84	1.53	3.59
1.87	4.50	1.69	4.06
2.91	5.34	2.81	5.31
2.81	8.62	1.69	8.06
* 2.31 ± .32	5.57 ± 1.2	1.93 ± .3	5.25 ± .1

* Mean ± S.E.

TABLE III. Effect of NAPA on Δ^1-17-OHCS* Plasma Levels in Rabbits following Prednisolone Administration.

Prednisolone dosage, mg/kg	Δ^1-17-OHCS plasma level: Without NAPA, Conj.,‡ μg/ml	Without NAPA, Free, μg/ml	With NAPA† Conj., μg/ml	With NAPA† Free, μg/ml	With NAPA† Free, % of control
10	1.31	2.26	1.22	4.59	203
10	1.22	2.24	1.78	6.47	289
10		1.31		4.97	394
10		3.28		4.41	134
10		3.78		6.09	162
Mean ± S.E.		2.56 ± .43		5.31 ± .40	236 ± 46
25	3.09	5.34	4.69	10.12	189
25	3.28	7.50	4.89	12.37	165

* Determined by Peterson modification(10) of Silber-Porter method(9).

† 250 mg/kg of body wt administered orally 30 min. before prednisolone.

‡ Released by β-glucuronidase hydrolysis.

Table III shows the effect of NAPA on plasma levels of 17-OHCS arising from prednisolone (Δ^1-hydrocortisone) administration. Two hours after concurrent administration of prednisolone (10 mg/kg) and NAPA (250 mg/kg) the mean 17-OHCS plasma level was 236 ± 46% of the control value. In 2 rabbits when the prednisolone dosage was 25 mg/kg the circulating 17-OHCS in the presence of NAPA averaged 177% of the control level. While there was an apparent slight increase in plasma level of conjugated 17-OHCS, the principal effect of NAPA was to increase the plasma level of free 17-OHCS.

Due to the fact that prednisolone does not react with INH to form a hydrazone it was not possible to measure the Δ^4-3-keto-corticosteroid component of the plasma 17-OHCS following prednisolone administration. However, it is interesting that after administration of prednisolone, no INH-reactive material was present in plasma, thus providing further evidence that the first step in prednisolone metabolism is not its conversion to cortisol by reduction of the Δ^1-bond.

Discussion. The increased plasma levels of

free 17-OHCS attained by concurrent administration of cortisol or prednisolone and NAPA are, in all probability, due to the fact that NAPA provides an alternative substrate, in high concentration, to the glucuronide-forming mechanism in liver, thus inhibiting formation of steroid-glucuronide.

Although both Porter-Silber and INH-reactive 17-OHCS in plasma show a similar increase after NAPA administration, there is no evidence that the substances measured in each case are completely identical. The Porter-Silber reactive materials may consist partially of tetrahydro derivatives of cortisol, which presumably are biologically inactive. However, it is by no means certain that these materials are not potentially biologically active, provided that their rapid conjugation with glucuronic acid and subsequent excretion is prevented, thus providing an opportunity for reoxidation by the adrenals.

The marked increase in plasma INH-reactive 17-OHCS in the presence of NAPA is not consistent with the widely held view that corticosteroid conjugation with glucuronic acid takes place only after the Δ^4-3-keto group has been reduced to a saturated 3-hydroxyl group (16,3). In this connection Weichselbaum and Margraf(17) have presented evidence which suggests that conjugation may take place at carbon atom 21 without previous reduction at the Δ^4-3-keto group. They also found that following beta-glucuronidase hydrolysis of human peripheral plasma, Δ^4-3-keto-corticosteroids appeared in considerable amounts, as measured by the INH reaction. This could account for the marked increase in plasma INH-reactive materials caused by NAPA administration. These materials are biologically active, or potentially so, since they contain the Δ^4-3-keto group.

Of some interest is the fact that no INH-reactive material was present in plasma following administration of prednisolone. This indicates that the Δ^1-bond of prednisolone is not reduced to yield Δ^4-3-ketocorticosteroids. Studies in humans(18) have shown that no excretion of cortisol or cortisone occurs as a result of prednisolone administration, and Vermeulen(19) points out that in all metabolites of Δ^1-corticoids so far identified, the dienone grouping was retained. Sandberg and Slaunwhite(20) found that at least 40% of an administered dose of prednisolone was excreted as glucuronic acid conjugates. Although the conjugates could not be identified, conjugation presumably occurred at positions other than carbon 3. In this case, the increased plasma 17-OHCS effected by NAPA administration would be comprised of corticosteroids with the dienone structure intact.

The results presented here suggest the possibility that physiological effectiveness of endogenous or exogenous steroids may be greatly increased by retarding their inactivation through the use of competitive glucuronylation inhibitors. Such studies are now in progress at the clinical level. Preliminary results as to the effect of NAPA on 17-OHCS plasma concentrations are in agreement with those obtained in rabbits.

Summary. Administration to rabbits of N-Acetyl-p-Aminophenol (NAPA) in conjunction with cortisol or prednisolone resulted in a marked increase in plasma levels of 17-OHCS. This effect is presumed to be due to inhibition by NAPA of steroid-glucuronide formation in the liver.

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Cultivation in Tissue Culture of the Virus of Avian Lymphomatosis. (23870)

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The demonstration by Burmester, Prickett and Belding(1) that a cell-free extract of the Olson(2) transplantable lymphoid tumor could transmit avian visceral lymphomatosis, firmly established the fact that an agent or agents other than cells could induce the disease. Further studies(3) have shown that filterable agents are present in other rapidly growing lymphoid tumor strains. Because propagation of viruses in various cell-medium systems is now common, it was thought that a system might be found in which the agent of avian visceral lymphomatosis would multiply and show its presence by induction of cytological damage. Previous attempts to do this have been only partially successful. Doljanski and Pikovski(4) have reviewed previous work and reported that they were able to propagate the agent of fowl leucosis in cultures of common mesenchyme cells but were unable to produce cytopathological changes.

The present communication is a preliminary report on successful propagation from avian lymphomatosis liver filtrates of an agent producing focal lesions in tissue culture, serial passage of the agent, infection of embryos and chicks with the cultured agent, and recovery of the agent from the diseased embryos and chicks.

Materials and methods. Virus. The virus was contained in a filtrate of lymphomatous liver obtained from Dr. B. R. Burmester, Regional Poultry Research Laboratory, East Lansing, Mich. The filtrate was designated RPL-12-L28 and represented the 16th serial filtrate passage in White Leghorn chicks. *Tissue culture.* Chick embryo liver cultures were prepared as follows: Sixteen-day-old chick embryo livers were minced and mildly agitated with 0.85% trypsin solution. Gross particles that settled rapidly were discarded. The remaining cells were then sedimented by low-speed centrifugation and washed with balanced salt solution. Tubes were seeded with approximately 500,000 cells in 1 ml of medium consisting of 30% horse serum and 70% medium 199(5), and incubated 2 days at 37°C. The medium was then replaced with 100% medium 199, after which the tubes were inoculated with tissue filtrates or infective tissue culture fluid. Cultures of whole chick embryo were prepared in a similar manner from 10- to 12-day-old embryos. A slightly modified Eagle(6) medium containing 10% horse serum was used. After 24 hours' incubation at 37°C, the medium was changed to 100% 199, and the tubes were inoculated. *Neutralization tests.* Serum neutralization tests in tissue culture were done by inoculating tubes with 0.1 ml of serum-virus mixture. A constant amount of virus (1,000 to 10,000 tissue culture infectious doses) was mixed with serial 2-fold dilutions of serum, and the mixture remained at room temperature for at least 2 hours. The neutralization titer of the serum was the highest dilution of serum in the incubated inoculum which prevented cellular destruction for 7 days.

Results. Tissue culture. Chick embryo liver cells grew on glass as discrete islands of hepatic cells surrounded by large strands of fibroblasts. On low power microscopic examination of living preparations, the first noticeable effect of the virus was swelling of he-

patic cells, some of which appeared to wander out of the islands. The cells showed increasing granularity and density of nucleus until cellular contours were lost. At this stage the cells often detached from the glass. Pathological changes were also observed at a later stage in the fibroblasts. These were essentially characterized by a diffuse granulation of cytoplasm giving the impression of sand scattered on the monolayer. That these changes were caused by the agent and not by toxic products from destroyed hepatic cells was shown by experiments with cultures of whole chick embryo cells. In such cultures the cell type is principally fibroblast. The agent induced pathological changes in these cultures which were similar, both in time of appearance and in microscopic characteristics, to those observed in fibroblasts of embryo liver cultures. When the inoculum was undiluted, very few cellular changes were evident in the first passage, but after the second or third passage the parenchymal cells showed characteristic changes within 48 hours. With diluted inocula, cytological lesions were not evident before the sixth day (144 hours). In the early blind passages, cultures were kept 10 or 12 days, or as long as cells of control tubes remained healthy. Incubation temperature was 37°C after adaptation, but cytological lesions occurred earlier at 39°C, and this temperature may be best for the first 2 or 3 passages. The infectious titer of culture fluid varied but $TC_{50}(7)$ titers as high as 10^9/ml have been obtained.

Other chick and chick embryo cells such as lung and kidney, and whole chick embryo cells (mostly fibroblasts) are attacked by the virus, but the most satisfactory results were obtained with embryonic liver preparations. Duck embryo cells were also susceptible but neither primary cultures nor established cell lines of mammalian cells would support growth of the virus.

Identification. Antisera to infectious bronchitis, Newcastle disease, laryngotracheitis, fowl pox and serum from normal chicks did not neutralize the agent, and the presence of pleuro-pneumonia-like organisms was not demonstrable in the cultures. Antisera to the agent prepared by inoculating chicks with infected tissue culture fluid had titers of 1:256. When an adjuvant was used with the culture fluid in preparation of immune sera, the titer was increased to 1:2048. Immune sera having similar titers were obtained when guinea pigs or rabbits were immunized. Two antisera to the lymphomatosis agent prepared by Dr. Burmester had titers of 1:256 and 1:512 respectively. Antisera prepared against normal chick embryo tissues and against Forssman antigen failed to neutralize the agent.

Immune sera to erythroblastosis and myeloblastosis were supplied by Dr. J. Beard, Duke University, and Dr. B. R. Burmester. The relation of these viruses to the agent under study was suggested by neutralization titers of 1:32 to 1:128 obtained with myeloblastosis antiserum, and 1:64 to 1:128 with erythroblastosis antiserum.

Infectivity. Chick embryo. Infection of embryos with this isolate was obtained regularly by the intravenous route and usually by the intracranial route of injection. Other routes of inoculation did not always produce infection. Best results were obtained with intravenous inoculation of 12-day embryos and death occurred in 3 to 7 days, depending on amount of virus introduced. Two types of lesions were observed. In the first 24 hours, lesions of 1 type were characterized by appearance in hepatic cells of large swollen nuclei containing numerous and conspicuous inclusion bodies. The hepatic cells became progressively dense, forming an amorphous mass in which no particular structures could be recognized. During this period no alterations were evident in other organs. It is interesting to note that identical chronological and morphological sequence of changes was also encountered in stained preparation of chick embryo liver tissue cultures infected with this virus. The second type of lesion was observed after 36 to 48 hours. The number of hepatic cells containing inclusions decreased, and round cells with a conspicuous vesicular nucleus and strongly basophilic cytoplasm appeared in the mesenchyme of various organs. These cells were particularly evident in the portal spaces surrounding the wall of vessels, but were also seen in interstitial connective

tissue of the myocardium, mesonephros and metanephros. The spleen volume was usually 2 to 3 times that of embryos inoculated with noninfectious material, and the large round cells constituted the greatest percentage of the cell population of the organ; correspondingly there was a depression of myeloid elements. Blood, bone marrow, yolk sac and *Fabricius bursa* of infected embryos showed no relevant changes. Three to 5 days after inoculation, the mortality was very high and at autopsy, concomitant to round cell infiltration, large areas of necrosis, often hemorrhagic, were observed in the liver.

Chicks. Chicks of White Rock stock known to have a low natural incidence of lymphomatosis were inoculated by various routes when 1 day to 3 weeks of age, with virus that had been propagated in either tissue culture or chick embryo. Virus that had gone through 8 or more serial passages was used to reduce the chance that the actual infectious agent had used the observed virus as carrier without itself being propagated in the process.

While there have been no cases of lymphomatosis observed in uninoculated birds, the response to inoculation has ranged from 15 to 56 percent. The best infection rate observed was 9 of 16 birds showing visceral lymphomatosis in a 9-month observation period. With the exception of 3 chicks which showed neural lymphomatosis, all had large livers in which the type of lymphomatosis varied from diffuse to nodular form.

Recovery of virus. Virus was readily recovered from infected chick embryos in titers which ranged up to 10^8 TCD_{50}/gram of embryo. Recovery of virus from adult lymphomatous livers has been less successful. A cytopathogenic agent has been found in most livers but some isolates induced infection only in fibroblasts of the culture.

Discussion. Avian visceral lymphomatosis in this report refers to the disease in chickens characterized by a round lymphoid type cell infiltration which is seen in intravascular infiltration or in extravascular nodules in liver or other organs. The spleen is usually greatly enlarged and in some cases primitive cell types may appear in the blood. Our work is interpreted to indicate that any one agent may cause various disease manifestations.

Although our isolate infected the chick embryo only by intravenous or intracranial route, some more recent isolates regularly induce infection of the embryo by inoculation of the yolk sac, allantoic cavity or chorio-allantoic membrane. There is considerable variation in behavior of all isolates in tissue culture.

Summary. An agent has been cultivated in tissue culture from avian lymphomatous liver filtrates which can be passed serially in chick embryo liver cells and which induces cytopathological changes. The agent induces in 12-day-old chick embryos a disease histologically resembling lymphomatosis. In chicks it has induced lymphomatosis in 20% to 55% of the birds in 9 months, and an agent similar to the original agent has been recovered from some of the diseased livers.

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Changes in Renal Function in Rats Drinking Heavy Water.* (23871)

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Among changes observed in rats drinking heavy water were increases in blood levels of NPN and urea(1). These increases, suggestive of impaired renal function, were apparent when about 15% of body water was replaced by D_2O; at 30% replacement, the lethal level, concentration of urea in blood was 4 times the normal value. Dybing *et al.*(2) reported that livers and spleens of mice treated with D_2O showed degenerative changes, but that kidneys were essentially normal. In rats, however, evidence of renal tubular damage has been observed histologically(1). This paper is concerned with the effect of heavy water on glomerular filtration and renal plasma flow in rats.

Methods. The rats used were Sprague-Dawley females, about 7 months old and weighing 250-280 g. They were given 50 mole % D_2O as drinking water, and received Rockland rat diet *ad libitum*. The concentration of D_2O in plasma was estimated by the method of Katz *et al.*(1). Studies of renal function were carried out essentially according to the method of Smith and Boss(3). Rats were given two 10-ml doses of water by stomach tube 50 minutes apart. The D_2O content of the water was adjusted to that of plasma. After each dose of water, 250 mg/kg of creatinine as a 5% solution, and 150 mg/kg of sodium *p*-aminohippurate (PAH) as a 1.5% solution in 2% sodium sulfate were injected subcutaneously. Immediately after second injection the bladders were emptied by suprapubic compression. The rats were then placed in metabolism cages and urine collected for 50 minutes. At this time, blood was taken from each rat by heart puncture, and plasma and urine were analyzed for creatinine and PAH. Volume of urine collected was recorded, although the values reported in Table I are consistently low because of incomplete drainage from the collection funnels. The analyses of course were carried out on urine combined with washings of the funnels. Measurements of renal function were made on 3 occasions before D_2O was given, and 6 times during the 38-day period when rats were drinking D_2O. After 3 of the rats died, the remainder were given H_2O to drink, and the studies were repeated 1 and 5 days later.

Results. Table I shows changes in urine output, creatinine clearance (C_{Cr}), and PAH clearance (C_{PAH}) in rats drinking D_2O. Within 9 days all had decreased significantly, and by the 36th day all were only about 40% of control values. Recovery of kidney function following restoration of H_2O was remarkably fast: even at 1 day after removal from D_2O a marked improvement in kidney function was apparent, and after 5 days all were actually slightly above normal values.

In another less extensive experiment we had previously observed similar changes in respect to C_{Cr}: at 28% replacement of body water by D_2O, glomerular filtration was reduced to 45% of control level; 8 days after restoration to H_2O, recovery was virtually complete (95%).

It is clear from Table I that the rats immediately ceased to gain weight when given D_2O, although extensive weight loss was observed only in 2 animals, both of which died. Deaths occurred on 33rd, 36th, 38th, and 40th days after beginning of experiment; the last death occurred after replacement of D_2O with H_2O. The other 4 animals apparently recovered completely. At no time during the study was a difference evident between rats that subsequently died and those that ultimately survived.

Discussion. The fact that both glomerular filtration (C_{Cr}) and renal plasma flow (C_{PAH}) decreased at approximately the same rate and to about the same extent, while D_2O concentration in the body was rising toward 30 mole %, suggests that there was no specific injury to the kidney *per se*. The rapidity with which animals recovered when transferred from D_2O to H_2O is also consistent with the idea that

* This work was performed under auspices of U. S. Atomic Energy Com.

TABLE I. Effect of D_2O on Kidney Function in Rats.

Days on D_2O	Plasma D_2O (mole %)	No. rats	Body wt (g)	Urine output (ml/min./100 cm² surface area) Avg	Urine output S.E.	C_{Cr} Avg	C_{Cr} S.E.	C_{PAH} Avg	C_{PAH} S.E.
−50		7	246	.033	.003	.521	.050	2.57	.20
−43		8	251	.027	.003	.616	.025	2.22	.06
− 2		8	266	.031	.002	.521	.027	2.54	.20
Avg pre-D_2O		23	—	.030	.002	.551	.022	2.43	.10
3	9.7	8	261	.022	.002	.592	.036	2.50	.17
9	18.2	8	265	.015	.001	.396	.028	1.53	.19
15	20.6	8	263	.014	.001	.372	.037	1.71	.17
22	23.2	8	260	.017	.002	.283	.045	1.25	.11
29	26.0	8	258	.017	.003	.249	.039	1.52	.23
36	28.3	6	249	.013	.002	.228	.017	.92	.21
After H_2O									
1	19.8	5	254	.026	.004	.434	.039	1.42	.24
5	<5	4	250	.032	.005	.628	.012	2.60	.29

the lesions observed microscopically may be of no significance insofar as the survival of the animal is concerned. It is also relevant to point out that none of 6 enzyme systems in the kidney, including the oxidative phosphorylation system, was significantly altered, even in moribund animals with 30 to 32% of their body water replaced by D_2O(1).

The elevated concentrations of urea and NPN and the decreased concentrations of plasma protein and glucose in blood of rats receiving D_2O(1) are strongly suggestive of adrenal insufficiency, since these changes were *quantitatively* similar to those seen in adrenalectomized rats(4). It has been shown in several species that reduction of renal function occurs after bilateral adrenalectomy (see Smith(5) for review).

Thus it seems likely that one of the reasons for the toxicity of D_2O is a disturbance in adrenal function. In agreement with this supposition are the observations that hypophysectomized rats, with atrophic adrenal glands, show toxic effects at much lower levels of D_2O, and that in normal rats the adrenals increase in size by as much as 50%(1). Whether adrenal hypertrophy is actually accompanied by an increase in hormonal production has not been established. The D_2O-treated rat may have a higher requirement for adrenal hormones than can be met even by a hyperplastic cortex; the requirement may be unaltered but production of adrenal hormones may be inhibited in deuterated tissues; or metabolic processes of the rat may be so altered by deuteration that no additional amount of adrenal hormones would permit the animal to adapt to the altered internal environment produced by D_2O.

Summary. Glomerular filtration and renal plasma flow were decreased to about 40% of normal in rats drinking 50 mole % D_2O. Upon transfer from D_2O to H_2O, renal function rapidly returned to normal. It is probable that the changes in renal function do not represent a direct toxic action of D_2O on the kidney, but are perhaps the result of disturbed adrenal function.

We wish to thank Dr. Henry L. Crespi and Mrs. Dolores Symon of Chemistry Division for measurements of D_2O in plasma, and for supplying triply distilled D_2O, and to thank Dr. J. J. Katz for his interest in this work.

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Purification of Intrinsic Factor.*† (23872)

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Intrinsic factor (IF) is a heat-labile substance found in normal human gastric juice and absent in persons with pernicious anemia. It has been demonstrated by using radioactive Vit. B_{12}, that the function of IF is to cause absorption of Vit. B_{12} from intestinal tract of man(2,3). Since hog stomach and hog duodenum contain IF activity, numerous investigators have been concerned with the purification and isolation of IF(4).

A highly purified intrinsic factor preparation obtained from desiccated hog stomach has been described(5). In our report, fresh hog duodenum was employed and an IF preparation termed RAS (reprecipitated ammonium sulfate) fraction, fully active at 5 mg, was obtained in 100 g quantities suitable for further purification. This RAS fraction was further purified by ion exchange on a cellulose derivative and an IF fraction active at 1 mg was obtained.

Method. Preparation of AS fraction. Hog duodenum was obtained at slaughter houses from freshly killed hogs. The outside of tissue was washed for a few seconds in water at about 30°. Loose adhering fat was removed from outside of tissue and contents of intestine, if present, were expelled manually. If the intestine was not processed for IF immediately, the tissue was quickly frozen in flat layers and kept frozen in a chill room at −10° until used. The duodenum in 10 kg batches was ground in meat grinder, mixed with 30 l of 2% NaCl solution for 30 minutes at 10-15°, then settled for 30 minutes. The suspension was filtered through 2 layers of cheesecloth. The material remaining on cheesecloth was re-extracted with 10 l of 2% NaCl solution for 15 minutes at 10-15° and allowed to settle for 15 minutes. This second suspension was also filtered through 2 layers of cheesecloth. The 2 extracts were combined and pH of combined extracts was adjusted to 9.0 with 1 N NaOH. After 30 minutes, the solution was adjusted to pH 1.5 with 1 N HCl. A precipitate formed and after 30 minutes was removed with Sharples centrifuge Model M-86-P-IE. The resulting supernatant was adjusted to pH 4.5 with 1 N NaOH and 194 g of $(NH_4)_2SO_4$/l of supernatant was added slowly with stirring. The solution stood for 4 hours and was then centrifuged in the Sharples centrifuge. The precipitate was discarded and to resulting supernatant, 174 g of $(NH_4)_2SO_4$/l of supernatant was added slowly with stirring. The solution was kept overnight and again centrifuged. The resulting supernatant was discarded and precipitate dialyzed in cellulose tubing to remove the $(NH_4)_2SO_4$. This fraction has been named ammonium sulfate (AS) fraction. Up to this point the procedure is a modification of the method of Prusoff *et al.*(6). *Preparation of RAS fraction.* The dialysate was then centrifuged at 15,000 times gravity for 15 minutes in angle head centrifuge at 2-5°. The precipitate was discarded and supernatant reprecipitated with $(NH_4)_2SO_4$ using 368 g/l. After standing overnight at 4° the precipitate was removed by centrifugation at the same temperature. The resulting precipitate was dialyzed and centrifuged at 15,000 times

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We also acknowledge the kind cooperation of Dr. R A. Brown, Research Division, American Cyanamid Co., for conducting physical chemical measurements, P. K. Sitteri, Research Division, for assistance in initial experiment on chromatography of DEAE-SF, and R. Smith for technical assistance.

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TABLE I. Evaluation of RAS Fraction in a Pernicious Anemia Patient in Relapse (Patient C.D.).*

Initial red blood cell count	2,510,000
Red blood cell count after 21 days	3,380,000
Reticulocyte peak	11.4%
Expected reticulocyte peak	11.0%

* This patient was given orally 5 mg of RAS fraction together with 10 μg of vit. B_{12}.

gravity to remove any insoluble material. The clear supernatant was dried from frozen state and termed reprecipitated ammonium sulfate (RAS) fraction. *Activity of RAS fraction.* RAS fraction was tested in a pernicious anemia patient in relapse (Table I), according to method of U.S.P. Anti-Anemia Board(7). It was also tested in pernicious anemia patients in remission (Table II) by modified

TABLE II. Determination of Activity of RAS Fraction by the Modified Urinary Excretion Test.

	% excretion in urine
RAS fraction (10 mg)	5.7
Reference intrinsic factor 186-1 (50 mg)	5.9

urinary excretion test(8,9), and at dosage of 10 mg caused excretion of as much Vit. B_{12} as 50 mg of the reference IF #186-1. It was therefore 5 times as potent as the reference intrinsic factor sample, #186-1. The reference IF #186-1 used was previously studied by Ellenbogen *et al.*(8), Williams *et al.*(10), Baker and Mollin(11), and Callender and Evans(12), and it was shown that 50 mg of 186-1 was equivalent to 2-3 daily oral doses. Therefore, RAS fraction is active clinically at daily oral dose of 5 mg. *Electrophoretic and sedimentation studies of RAS fraction.* Electrophoretic analyses in phosphate-saline buffer of pH 6.1 and 0.1 ionic strength and acetate buffer of pH 4.1 and 0.1 ionic strength at

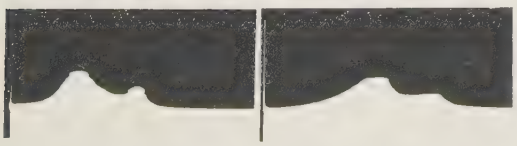


FIG. 1. Descending electrophoretic diagrams of RAS fraction. Left: Electrophoresis performed in phosphate-saline buffer of pH 6.1 and 0.1 ionic strength. Protein conc., 1.2%. Time of exposure, 80 min. Right: Electrophoresis performed in acetate buffer of pH 4.1 and 0.1 ionic strength. Protein conc., 1%. Time of exposure, 80 min.

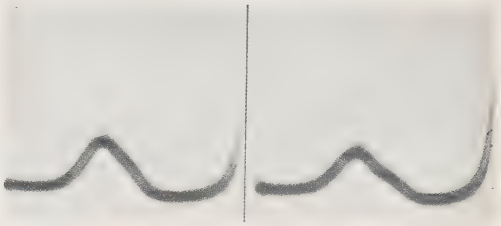


FIG. 2. Sedimentation diagram of RAS fraction. Ultracentrifugal analysis in Spinco ultracentrifuge at 59,780 rpm using synthetic boundary cell. Phosphate buffer pH 6.4, .002 ionic strength, with saline 0.14 ionic strength. Left: 24 min. Right: 32 min.

4° revealed that RAS fraction was heterogeneous (Fig. 1). Migration of components was towards opposite electrodes at these 2 pH ranges, indicating that IF present in RAS fraction had an isoelectric point between pH 4.1 and 6.1. Ultracentrifugal analysis in Spinco ultracentrifuge at 59,780 rpm with a synthetic boundary cell indicated that RAS fraction contained at least 2 major components, one with low sedimentation constant of about 0.7 S. (Fig. 2). To separate the high molecular weight (HMW) component and the low molecular weight (LMW) component of RAS fraction, centrifugation was carried out at 100,000 times gravity at 4° with Spinco Model E ultracentrifuge. In an initial attempt, centrifugation was carried out for 37 hours resulting in approximately 2-fold concentration of LMW component, although there was definite activity in both the LMW and HMW components. Centrifugation for 64 hours resulted in concentration of activity of HMW component. It is possible that intrinsic factor activity was associated with a component which could not be observed by ultracentrifuge analysis. In no instance was separation of components complete. *Cellulose ion-exchange purification of RAS fraction.* The anion exchange cellulose absorbent DEAE-Solka Floc (DEAE-SF)—100-200 mesh—was prepared according to methods of Peterson and Sober(13,14) and used to separate RAS fraction into 3 fractions. One hundred mg of RAS fraction was dissolved in 3 ml of 0.025 M phosphate buffer, pH 7.5, and applied to top of 1.4 x 24 cm column and allowed to run into the adsorbent by gravity. Three 1-ml portions of the buffer were used

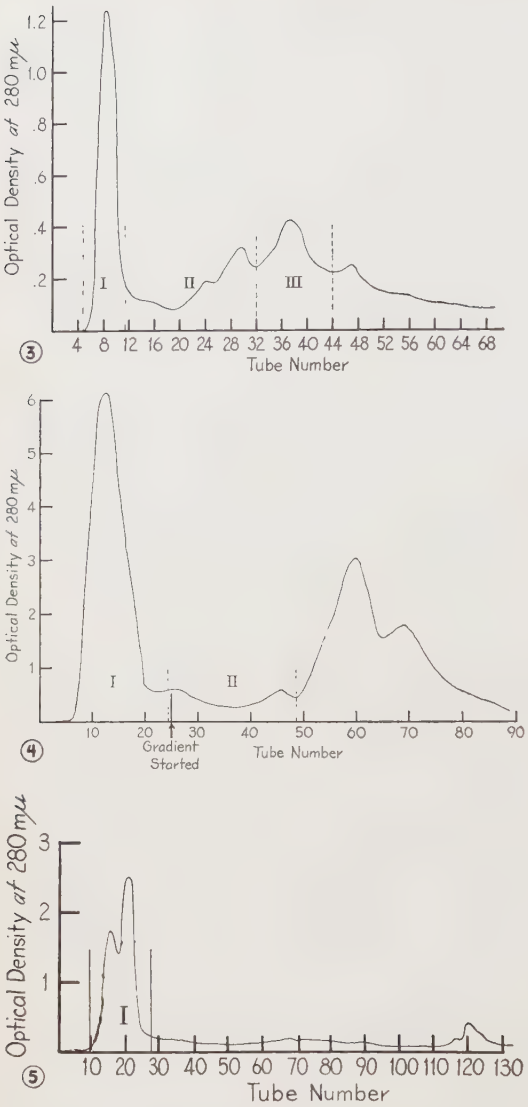


FIG. 3. Effluent diagram of 100 mg of RAS fraction on 1.4 × 24 cm column. See text for conditions of chromatography.

FIG. 4. Effluent diagram of 2 g of RAS fraction on a 2.8 × 32 cm column. First buffer (0.025 M phosphate—pH 7.5) was used until tube 25. pH gradient was started at tube 25 with pH 5.5 NaCl phosphate buffer.

FIG. 5. Rechromatography of column fraction II on 2.5 × 26 cm column. pH gradient was not started until tube 40. First buffer (0.025 M phosphate, pH 7.5) used as effluent until tube 40. First peak contained 52.7% of O.D. units of combined column fraction II before rechromatography.

to wash the sides of column after protein solution was added. A pH and ionic strength gradient was established with pH 5.5 NaCl-phosphate buffer (0.2 M NaH_2PO_4 and 0.2 M NaCl) and allowed to run by gravity with a 10 to 40 in. buffer head. Elution was carried out in chill room at 4°. Flow rates were 4 ml/hr and 4 ml/tube were collected with the aid of a drop counter. The effluent fractions were examined in a Beckman DU spectrophotometer at 280 mμ.

Results. Fig. 3 shows an elution diagram obtained when RAS fraction was chromatographed on DEAE-SF. Chromatographic components were divided into 3 fractions as indicated by dotted vertical lines. Tubes from each of these 3 fractions were combined, dialyzed and lyophilized and then tested by the modified urinary excretion test(8,9). Results of assays are shown in Table III. Both fractions I and III showed little if any activity in urinary excretion test at 10 mg whereas fraction II representing about 20% of total protein of effluent showed full activity at 3 mg which should be equivalent to clinical activity at 1 mg (17 times as active as 186-1).

Larger amounts of RAS fraction (2-4 g) were chromatographed on 2.8 x 32 cm columns. The large inactive fraction (I) which came off the column first, emerged even before a pH gradient was established with pH 5.5 buffer (Fig. 4). As with smaller columns, intrinsic factor activity was present far down the descending slope of column fraction I and continued through the middle section of the curve (column fraction II). This middle section of the effluent diagram was somewhat flatter in these larger columns than column fraction II obtained in the smaller column (*cf.* Fig. 3).

Recovery of protein as measured by optical density was usually between 90 and 100%.

Column fraction II from 8 additional chromatographic separations, totaling 4.1 g, were combined and rechromatographed on DEAE-SF. Fig. 5 shows the effluent diagram obtained on rechromatography of fraction II. Activity of rechromatographed fraction II was present in the first peak this time, even though the pH gradient was started well past the flat portion of effluent diagram. No further increase in potency, however, was achieved.

TABLE III. Assay of Fractions from Cellulose Ion-Exchange by the Modified Urinary Excretion Test.*

Material	Dose (mg)	% excretion in urinary excretion test: Vit. B_{12} Co^{60} only	Vit. B_{12} Co^{60} + fraction	Vit. B_{12} Co^{60} + 50 mg of reference IF sample, 186-1
Column fraction I	10	.4	1.9	12.3
II	10	.4	19.2	12.3
II	3	.7	6.8	6.7
III	10	.4	2.8	12.3

* See effluent diagram in Fig. 3.

In the ultracentrifuge the active peak from rechromatography appeared heterogeneous (Fig. 6). Approximately 70% of this material showed a sedimentation constant of 2.9 S, 15% with 1.0 S and 15% with 11 S.

Discussion. One of the main difficulties in fractionation of IF has been in obtaining large quantities of a preparation of intermediate potency, *i.e.*, about 5 times as active as the reference sample #186-1. Preparation of RAS fraction represents a solution to this problem.

This RAS fraction prior to chromatography on DEAE-SF is about twice as potent as the IF obtained by Robbins and Shields(15) after purification on cellulose ion exchanger.

In regard to number of daily oral doses of IF necessary to give near maximum urinary excretion test, Robbins and Shields(15) have failed to note that some of the hematological data presented by Williams *et al.*(10) were after only 14 days of treatment. They have also failed to note that Baker and Mollin(11) found that 50 mg of IF caused absorption of 0.5 μg or more of Vit. B_{12} from 0.8 μg dose. When tested in the same patient, 50 mg of IF #186-1 caused excretion of 0.452 μg of Vit. B_{12} whereas 50 mg of standard used by Robbins and Shields caused excretion of 0.230 μg of Vit. B_{12} with a 2 μg oral dose.

Preliminary chromatographic experiments of RAS with DEAE-SF for further purification of IF have resulted in a 5-fold concentration. However, failure to obtain further purification upon rechromatography of column fraction II (Fig. 5) may indicate that further changes in pH, ionic strength or elution schedules may be necessary to obtain further resolution. All investigations thus far have been conducted using sodium phosphate buffers. It is quite possible that other buffer systems will provide better resolution. In view of the fact that the active peak after rechromatography contained 52.7% of total O.D. units, a 2-fold increase in potency is the best that can be obtained under conditions of this rechromatography.

It is quite likely that active fraction II of original chromatograms was displaced backwards by presence of fraction I, since in absence of fraction I (rechromatography of combined fraction II) the active fraction emerged as a sharper and more concentrated peak (Fig. 5).

Bacterial contamination was a problem frequently encountered in the use of larger amounts of IF for chromatography. Reluctance to freeze and thaw individual tubes for dialysis, assay, rechromatography and assay again necessitated exposure of dilute solution to 4° for as long as one week. Methods of preventing bacterial contamination are presently being investigated. Preliminary studies indicate that use of sterile distilled water and autoclaved glassware appear beneficial.

Another probable loss of activity has resulted from dialysis and lyophilization of very dilute solutions. That changes have taken

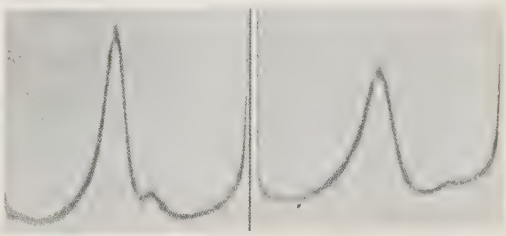


FIG. 6. Sedimentation diagram of column fraction I obtained from rechromatography of column fraction II on DEAE-SF. Ultracentrifugal analysis in a Spinco ultracentrifuge at 59,780 rpm using a synthetic boundary cell. Left: 8 min. Right: 16 min.

place after dialysis and drying is shown by the fact that dried IF was not completely soluble when an attempt was made to redissolve it. It is noteworthy that IF clinically active at 1 mg was heterogeneous. Unless there is more than one substance that can act as IF, this would indicate that pure IF is physiologically active at less than 1 mg.

Summary. A highly purified intrinsic factor preparation (RAS fraction) was prepared in quantity from fresh hog duodenum by saline extraction, pH adjustment and ammonium sulfate precipitation. This fraction was active in pernicious anemia patients in relapse at daily oral dose of 5 mg. Ultracentrifugal separation could not completely separate the components of RAS fraction. RAS fraction was further purified on a DEAE-cellulose ion exchanger to give a fraction which was active in urinary excretion test at a dose of 3 mg, equivalent to a daily oral dose of 1 mg in pernicious anemia patients in relapse. Rechromatography of this active fraction resulted in no further increase in potency. Ultracentrifugal studies of the most active fraction obtained from rechromatography showed essentially 3 peaks with sedimentation constants of 1.0 S, 2.8 S and 11 S.

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Effect of 17-Ethyl-19-Nortestosterone on Hyperglycemic Action of Glucagon in Humans. (23873)

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There are only a few known conditions in which human subjects will fail to respond to intravenous injection of pancreatic hyperglycemic factor (HGF or glucagon) with substantial rise in blood sugar. These are: a) if liver glycogen is low, as in starvation, severe liver disease or diabetic acidosis, and b) if liver glycogen is abundant but a specific enzyme deficiency prevents glycogenolysis, *e.g.* deficiency of glucose - 6 - phosphatase in glycogen-storage disease(1).

A chance observation in one patient led to the suspicion that 17 ethyl-19-nortestosterone (Nilevar†) might depress glucagon-in-

* With technical assistance of Miss Grace Hughes and Mr. Stuart Marcus.

† Kindly supplied by Dr. Robert L. Craig, G. D. Searle and Co., Chicago, Ill.

duced hyperglycemia. Investigation of the effect of Nilevar on hyperglycemic response to glucagon was thus undertaken.

Methods. A total of 16 patients was studied. Eight were being treated with Nilevar when the study was instituted. At least one glucagon tolerance test was done on each patient while under therapy. When possible one or more tests were done at varying intervals after therapy had been stopped. The other 8 patients were treated with Nilevar after control glucagon tests were done. Glucagon response was then studied at repeated intervals while treatment continued. Most patients received Nilevar for relief of osteoporosis associated with some chronic illness, or because an anabolic hormone was indicated. Their nutritional status and daily food intake were fair to good. There were 2 males and 14 females with age range of 41-80 years. Three women were diabetics. Usual dose of Nilevar was 10 mg 3 times a day by mouth. In 2 patients this was later doubled, and 2 patients were given twice this dose from the beginning. One patient received only 10 mg twice a day. Glucagon tolerance test was done as standardized in this institution by several hundred tests. After 14-17 hour fast, 2 control blood specimens were taken at 10-minute intervals. 0.1 mg crystalline glucagon‡ was injected intravenously and blood specimens drawn 10, 20, 30, 45, 60, 90 and 120 minutes later. All were 0.1 ml samples taken in duplicate from finger blood. Blood sugar was determined by micro method of Somogyi as modified by Nelson(2). In 4 instances, patients were tested with 2 or 5 times the usual dose of glucagon. Twenty-four hour thyroid I^{131} uptakes were done on 8 patients while on Nilevar therapy.

Results. Table I summarizes results in the 16 patients. Nilevar produced a highly significant uniform depression of the hyperglycemic response to glucagon. Suppression of response to glucagon was complete in all patients except 3 (R.G., K.L. and L.V.) who received the drug only 8 days or less. From a large experience with this test, the lower limit of normal response to 0.1 mg glucagon given intravenously is a rise in blood sugar of 20 mg %. A rise of 15 mg % or less is considered a negative or absent response, 16-19 mg % being borderline.§

TABLE I. Maximum Blood Sugar Response to 0.1 mg H.G.F. Intravenously (Increase in Blood Sugar in mg %).

Patient	Off Nilevar	On Nilevar	Duration of Nilevar therapy at time of test
R.B.	33	13	9 days
R.B.	108	8	71 "
L.D.	70	0	67 "
E.E.		7	2 mo
R.G.	71	41	4 days
D.G.		11	3 mo
I.K.		5	2 "
J.K.	81	0	28 days
K.L.	57	20	8 "
I.P.	50	0	4 mo
B.P.	36	14	7 days
E.R.	29	7	2½ mo
L.V.		26	2 days
I.W.	101	12	17 "
F.W.	70	14	8 "
R.W.		0	34 "
*	64.2 ± 26.4	11.1 ± 10.9	
P		<.003	

* Mean ± S.D.

On a dose of 30 mg/day, beginning suppression was evident as early as 4 days in some patients. Suppression was complete by 9 days to one month in patients tested repeatedly. In 2 instances (R.B. and L.D.), the dose had to be doubled to 60 mg/day before negative tests were obtained. The 2 patients (B.P. and P.W.) given 60 mg/day initially showed absent glucagon responses more rapidly, *i.e.*, in 7 and 8 days respectively. 0.2 mg and 0.5 mg glucagon i.v. failed to change negative glucagon responses to positive ones.

Zero responses indicate no rise at all. Instead, blood sugars went below initial levels as much as 18 to 33 mg %. For statistical purposes, it was considered more valid and more significant to call these "zero" rather than "minus" responses. Had the latter been done, the difference between means would have been greater.

Table II demonstrates that mean response of Nilevar treated patients to glucagon is significantly different from that of previous large

‡ Kindly supplied by Dr. W. R. Kirtley, Lilly Research Laboratories, Indianapolis, Ind. Lot No. 0357-P-6046.

§ From unpublished data.

TABLE II. Maximum Blood Sugar Response to 0.1 mg H.G.F. Intravenously (Increase in Blood Sugar in mg %).

Condition	No. of patients	Mean	S.D.	P
Control*	37	51.4	12.8	
On Nilevar†	16	11.1	10.9	<.003

* Previous study. † This study.

control series of similar patients.

Fig. 1 demonstrates some typical curves of response to 0.1 mg HGF intravenously. As seen in Ic, the glucagon response returns toward normal shortly after Nilevar is discontinued.

The 24-hour thyroid I^{131} uptakes varied from 21-55%, only one being outside normal range, which for this laboratory is 15-50%.

The control of diabetic state was not obviously altered by Nilevar therapy in the 3 patients under observation. There was no evidence of hypoglycemia in non-diabetic subjects.

Discussion. It is apparent that Nilevar inhibits normal hyperglycemic response to glucagon. How this occurs is speculation. The first consideration is that Nilevar is a potent protein anabolic substance(3). Glucagon, on the other hand, has been shown recently to increase gluconeogenesis and to be protein-catabolic in a manner similar to, but independent of adrenal glucocorticoids(4). Does Nilevar block glucagon-induced hyperglycemia by preventing the enhanced gluconeogenesis and protein catabolism? If so, this would indicate that under certain circumstances, gluconeogenesis may be a more important mechanism of glucagon-caused hyperglycemia than liver glycogenolysis.

The fact that hyperglycemic response to glucagon is completely abolished only after many days or weeks of Nilevar therapy suggests liver glycogen depletion. Pretreatment with adrenocorticotrophic hormone (ACTH), cortisone or somatotrophic hormone (STH) enhance the hyperglycemic response to glucagon, presumably because they increase liver glycogen deposition(1). Since Nilevar has progestational and androgenic activity(3,5), it may inhibit pituitary and hence adrenal gluco-corticoid secretion. This might result in decreased liver glycogen and diminished hyperglycemic response to glucagon, which

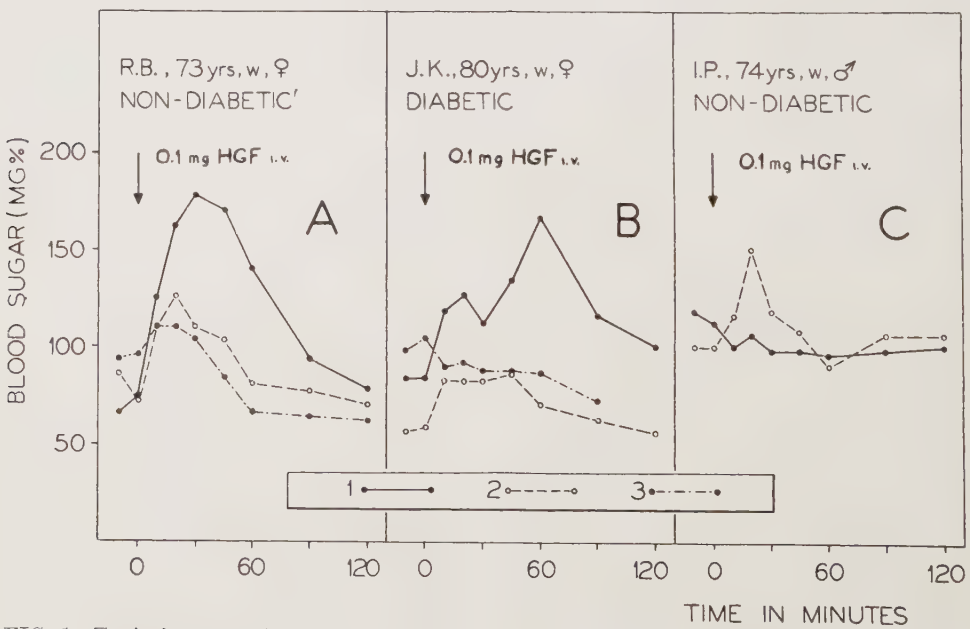


FIG. 1. Typical curves of response of blood sugar to 0.1 mg HGF intrav. before and during Nilevar therapy. A. Curve (1) before Nilevar; (2) 30 mg Nilevar/day for 12 days; (3) 30 mg Nilevar/day for 56 days and 60 mg/day for 6 more days. B. Curve (1) 30 mg Nilevar/day for 1 day; (2) 30 mg Nilevar/day for 12 days; (3) 30 mg Nilevar/day for 28 days. C. Curve (1) 30 mg Nilevar/day for 4 mo; (2) 12 days after Nilevar discontinued.

acts primarily by increasing liver glycogenolysis(1). Unpublished data on 5 patients in this hospital‖ demonstrate that Nilevar therapy does not alter plasma gluco-corticoid levels or ACTH responsiveness of adrenal glands. The normal I^{131} uptakes of thyroids of patients in our study are also indirect evidence of lack of pituitary inhibition by this drug. However, this does not rule out the possibility of direct interference by Nilevar of gluco-corticoid or other hormonal enhancement of liver glycogen deposition.

Both mechanisms may contribute to our findings, *i.e.*, diminished liver glycogen and direct antagonism of anabolic and catabolic substance. Nilevar, by decreasing gluconeogenesis by glucagon or adrenal glucocorticoids may gradually deplete the liver of glycogen. There is the possibility that Nilevar is an enzyme poison or inhibitor. This is of course compatible with any of the above mentioned theoretical mechanisms. Finally, Nilevar may produce this alteration of liver function by causing actual liver cell damage. The rapid return to a normal glucagon response in all patients after Nilevar was discontinued, argues against this.

Whatever the mechanism(s) may be, there exists the intriguing thought that therein may lie a clue to the role of glucagon in normal body economy.

Summary. Sixteen patients were studied as to the effect of Nilevar on hyperglycemic response to glucagon. Nilevar abolished or profoundly diminished this response. This effect was reversible when Nilevar therapy was stopped. Nilevar had no apparent effect on the control of 3 diabetic patients nor on the 24-hour thyroid I^{131} uptakes of 8 patients. Possible mechanisms are discussed.

‖ Studied in collaboration with Dr. M. G. Goldner and Dr. A. Carter.

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Strain Gauge Measurement of Tension Changes in Rat Colon.* (23874)

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In both rat and dog, we have found a direct association between blood pressure changes and movements of sodium into and out of extracellular compartment(1,2). Our current interpretation of these and other results is that blood pressure regulation depends on sodium transfer systems, broadly defined, which govern rate of sodium exchange between vascular smooth muscle and the surrounding medium. Definitive proof of this theory depends on study of the response of smooth muscle (vascular and other) to acute alterations of the cationic medium, or on analysis of cationic changes in the medium during and after induction of contraction in the muscle strip.

Methods currently in use for study of smooth muscle *in vitro* are unfortunately not applicable to this problem, since they would not permit rapid analysis or rapid alteration of the medium and further, the high volume of the medium relative to tissue would tend to dilute sodium shifts beyond detection limits of current methods. Thus, we have prepared new instruments. These have turned out to be simple, rugged and highly sensitive; further, they seem to us to be generally useful for study of smooth muscle strips. The present

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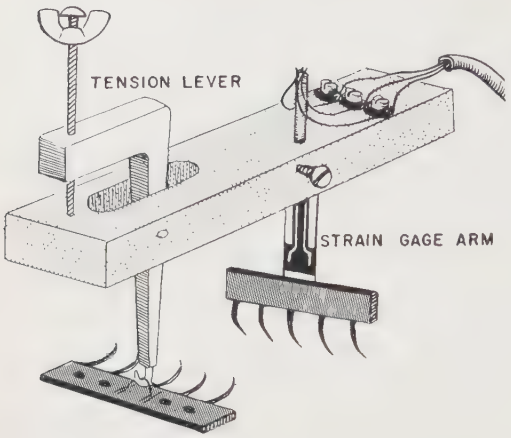


FIG. 1. Type I Tensometer. The body (10 × 2 × 1 cm) is lucite; other parts are brass, varnish-coated for insulation. Two strain gauges (Type A8, Baldwin, Lima, Hamilton) are mounted back-to-back on phosphor-bronze. The instrument is supported on a vertical rod with standard clamps for simple raising and lowering.

report deals with rat colon as test tissue and, as a by-product, the usefulness of this particular tissue is demonstrated. *Type I Tensometer* (Fig. 1) is a self-contained immersion unit for use with exchangeable baths, thus ensuring rapid exchange of medium. *Type II Tensometer* (Fig. 2) is an adaptation of the immersion unit featuring volume and temperature control of bath and a closed external pump circuit permitting rapid sampling of medium. This latter tensometer is also suitable for continuous recording of ion concentration using radioactive tracer technics. Both instruments feature back to back mounting of the strain gauges. These are connected into 2 arms of the bridge circuit of a Sanborn strain gauge amplifier recording through a Sanborn direct writing oscillograph. The double gauge feature results in high gain and low drift. *Basic sensitivity* trials reported below were carried out using the simpler Type I instrument with bath at room temperature. Test tissue was taken from distal 7 cm of colon from adult male albino rats of inbred Wistar strain. The colon was split longitudinally and placed in buffer solution for at least 30 minutes. Two strips approximately 3 cm in length were mounted (total weight approximately 0.5 g) and left immersed for 90 minutes more before use. A Krebs-Henseleit medium(3), modified for the rat, was used. Its composition in mM/liter is as follows: NaCl 129, KCl 5, $CaCl_2$ 2.1, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 200 mg %. The buffer was continuously aerated with carbogen to pH 7.4. The bath volume was 70 ml, mixed constantly by magnetic stirrer. *Tension* was measured as cm deflection of stylus calibrated as μin/in strain. For our Type I instrument used, 14 μin/in strain = 1 g tension on direct calibration. The attenuator built into the amplifier permits a rapid adjustment of amplification in steps for easy full-scale reading. Within the tension range the response was linear.

Results. a) Carbachol. Measured amounts of carbachol (carbamylcholine) dissolved in 0.2 ml of distilled water were added to the bath to cover a wide range of final concentrations and the isometric tension response was measured. The threshold dose for 8 separate pieces of tissue ranged from 3 to 4 mμg (mμg = 10^{-9} g) of drug/ml of medium. The dose response curve for 3 typical runs is shown in Fig. 3A. Responsiveness of tissue was increased if, within limits, a small basal tension was applied (up to 40 μin/in). This is shown for 3 separate pieces of tissue in Fig. 3B, indicating the necessity for standardizing the basal tension in quantitative studies. Unless otherwise noted the basal tension in these studies was 10 μin/in applied

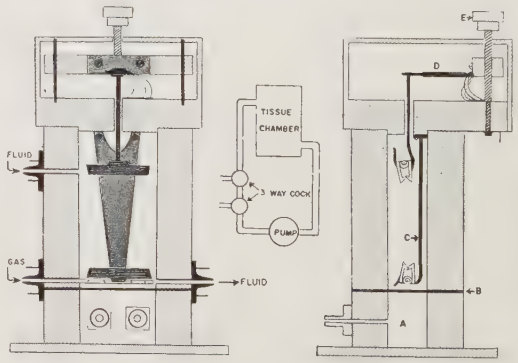


FIG. 2. Type II Tensometer showing external closed circulating system with a small centrifugal pump as driver. Stippled parts are lucite; other parts are metal, varnish or plastic-coated for insulation. A, heat exchange chamber; B, copper heat exchange plate fully insulated from upper chamber; C, rigid support arm; D, strain gauge arm; E, basal tension adjustment.

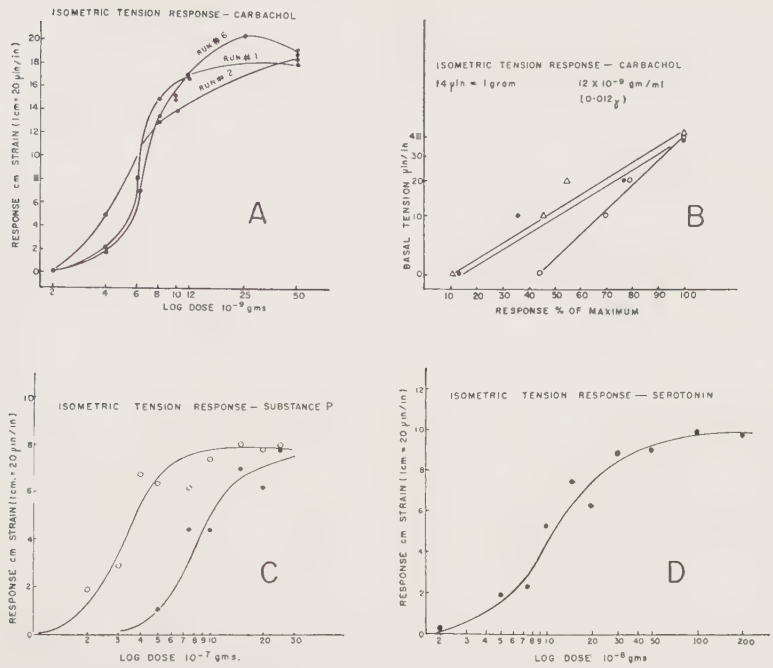


FIG. 3. A. Dose response, carbachol. B. Effect of altered basal tension on response to carbachol. C. Dose response, Substance P. D. Dose response, serotonin creatinine sulfate.

at start of experiment and left unchanged.

b) *Substance P* (*vonEuler*). An amount of this material sufficient for 2 trials was available. The dose response curves are shown in Fig. 3C. Potency of the material was approximately 60 vonEuler units/mg so that the threshold response ranged from 0.01 to 0.03 unit. The latent period, as shown in tracings of Fig. 4, was as described by Pernow for this substance(4).

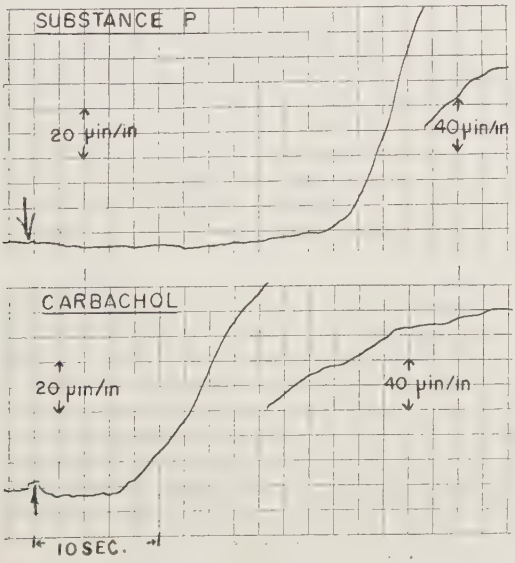


FIG. 4. Comparable responses to Substance P and carbachol showing latency periods.

c) *Serotonin.* This material was used as serotonin creatinine sulfate and doses are expressed in terms of the salt. The results for several separate pieces of tissue with replicate runs in each were quite stable. A typical dose response curve is shown in Fig. 3D.

Discussion. Sensitivity data for the strain gauge tensometer using rat colon strips have been presented. Absolute comparisons are difficult since rat colon has not been much used for this type of work. Some figures, however, are of interest. Thus, Innes *et al.* (5) found that 5 to 13 mμg/ml of carbachol produced tension changes of 1 to 5 g in guinea pig ileum; in our work, the threshold for rat colon was 4 mμg/ml while 13 mμg/ml produced strain in the range of 15 g tension. For Substance P and rat duodenum Pernow(4) reported that the threshold was 2 units/ml and for guinea pig ileum 0.1 unit/ml; we found the threshold for rat colon to be 0.01 to 0.03 unit/ml. For serotonin (measured as base) and atropinized rat colon the reported threshold

was 0.01 to 0.02 μg/ml and with time the threshold decreased 10-fold(6); in our work the threshold for non-atropinized rat colon was 0.02 μg/ml of salt (approximately 0.01 μg/ml as base).

It should be noted that our measurements were obtained at room temperature, a point of considerable convenience, although presumably the sensitivity would be greater at 37°. Rat colon is thus a useful tissue for such studies.

Summary. Strain gauge tensometers applicable to the study of ionic or metabolic exchanges in smooth muscle strips have been developed. Sensitivity was assessed using rat colon with carbachol, Substance P, and serotonin. The threshold for carbachol was 4 mμg/ml, for Substance P 0.01 to 0.03 unit/ml (vonEuler units) and for serotonin as salt 0.02 μg/ml.

We are indebted to Professor U. S. von Euler who graciously supplied Substance P.

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Effect of Malonate on Performance and Metabolism of Isolated Dog Heart.* (23875)

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Malonate is the classical inhibitor of the Krebs tricarboxylic acid cycle. Krebs and Eggleston(1), using pigeon breast muscle mince, found that 0.025 M malonate caused 92% inhibition of pyruvate oxidation. Smyth (2) used the same concentration of malonate in sheep heart mince and observed an 80% inhibition of pyruvate oxidation. If malonate were added in sufficient concentration to dog heart-lung preparation one would expect to produce heart insufficiency. For if it is assumed that oxidative metabolism in the heart is mainly channeled through the Krebs cycle, an interruption of the latter would lead not only to inhibition of oxidation, but also, according to current concepts, to cessation of production of high-energy phosphate bonds which, directly or indirectly, provide energy for muscle contraction. Early experiments with 0.03 M malonate produced a mild insufficiency lasting a few minutes, followed by a return to normal performance. In a few instances, there was even a "rebound" and a greater heart output was observed than before malonate addition. This unexpected result could have several explanations, 1) that malonate did not diffuse into heart muscle in sufficient concentration to inhibit succinate oxidation. Succinate determinations were then performed on malonate-treated hearts, an accumulation of succinate being interpreted as evidence for diffusibility of malonate.

Methods. Dog heart-lung preparations were performed under pentobarbital anesthesia but blood donors were anesthetized with chloroform. 5.6 g Na_2 malonate H_2O were dissolved in 50 cc H_2O and added to venous reservoir in 10 minutes. This brings molarity of the system to 0.03, if total weight of preparation is taken as 1 kg. Where double dose was used 5 minutes elapsed and another dose of 5.6 g malonate in 50 cc water was introduced in 20 minutes. Experiments were terminated 65 min. after onset of first malonate infusion. Phosphorus compounds were estimated as previously described(3) except

* This study was supported by grant from Am. Heart Assn.

that 7 instead of 10-minute hydrolysis in 1N HCl at 100°C was used to split off two-thirds of ATP-phosphorus. Phosphorus values are expressed as percentages of total acid-soluble phosphorus. By coincidence, the average total acid-soluble phosphorus is just a little over 100 mg phosphorus/100 g heart muscle tissue. Succinic acid was determined by enzymatic method of Szent-Györgi and Gozsy(4). However, some important precautions must be taken. a) Permanganate to destroy malonate should be applied not to original protein-free filtrate, as in earlier work of Krebs(1), but to the residue of ether extract derived therefrom, otherwise too high values for succinate are obtained. In dog serum, the difference can be of the order of several hundred percent. b) Perfect recovery of succinate is obtained if added to protein-free filtrate of muscle, but if added to a chilled aqueous suspension of muscle powder before deproteinization, recovery is incomplete. Recovery can be improved to 85-90% if the aqueous suspension containing added succinate is well mixed with excess sulfuric acid before tungstate is added. This procedure was mainly followed. Addition of succinate to sulfuric acid solution before introduction of tissue and tungstate leads to satisfactory recovery. Excised piece of heart tissue is frozen quickly in CO_2-snow, weighed, then pulverized in CO_2-snow. The powder is transferred quantitatively to glass-stoppered flask containing 7.75 ml glass-redistilled water and 0.8 ml 1:1 H_2SO_4/g of tissue. Shake well for 2 minutes then add 0.5 ml 10% sodium tungstate and continue shaking in the cold 10 minutes. Centrifuge and use 15-20 ml for extraction with ether in Kutscher-Steudel apparatus. The ether extract, to which 0.6 ml 0.1 M phosphate buffer of pH 7.4 is added, is evaporated and last traces of ether are removed by evacuating in desiccator for 15 minutes. Transfer to Kutscher-Steudel extractor using a total of 12 ml warm water, add 1 ml 1:1 H_2SO_4 and 2 ml 5% permanganate and mix. Place in water bath previously heated to 80°C for 30 min. at end of which water cools off to 50°C. Add another 1 ml 1:1 H_2SO_4, mix and add ether gradually while mixing. Again, 0.6 ml phosphate buffer is added to ether extract and the ether distilled off. Neutralize aqueous residue with NaOH using phenol red. Dry completely in vacuum desiccator over H_2SO_4 and take up the residue in 0.6 ml warm water. Use 0.3 ml of solution for enzymatic determination with succinoxidase.

Results. The "resting" succinate values in heart tissue, as determined in open-chest animals with artificial respiration show considerable variation. In 12 animals, the values ranged from 0.0 to 0.12, an average 0.05 $\pm$ 0.011 mg succinic acid/g wet tissue. In 8 control heart-lung preparations for 65 min. the average succinic acid content was 0.06 $\pm$ 0.013 mg/g (Table I). In 0.03 M malonate preparations the succinic acid content was 0.243 $\pm$ 0.033 mg/g. The difference is significant. The succinate accumulation with double dose of malonate is 0.29 $\pm$ 0.016. Succinate values reported by Krebs and Eggleston(1) and Smyth(2) are probably too high since these authors did not use double extraction procedure. These results do not prove, but constitute presumptive evidence that malonate diffused into heart muscles. Experiments by Busch and Potter(9) on intact rats provide direct evidence that malonate freely diffuses into rat heart *in situ*.

Table I shows that up to one hour, 0.03 M malonate had no significant effect on performance of the isolated heart as judged by changes in minute output and venous pressure. Only during addition of malonate, there was a transient mild insufficiency, probably not specific for malonate since it can also be induced by succinate. In 5 malonate experiments, Krayer's "competence index" (5) was also measured at beginning and end of experiment and in 4 of these, the index remained unchanged. Only in one case was there a change from 0.9 to 0.7. There is no correlation between succinate accumulation and heart performance, or between succinate accumulation and level of high-energy phosphorus compounds, which also remained within normal limits.

The results obtained with double dose of malonate (0.06 M) are partly reproduced in Table I. Before infusion of second dose was complete, there was in each case a sudden dilatation of the heart with significant de-

TABLE I. Effect of Malonate on Performance and Metabolism of Dog Heart-Lung Preparation. Duration of experiments, 65 min. (Phosphorus values expressed as % of total acid-soluble phosphorus.)

% change in systemic output at end of exp.	Change in venous pressure at end of exp. (cm of H_2O)	Succinic acid, mg/g heart tissue	Phospho-creatine-P	Inorganic-P	Labile nucleotide-P
		Controls			
−20	+ .7	.04	24.7	13.0	31.0
0	+ .1	.08	23.1	16.2	31.2
0	.0	.09	18.4	16.0	29.6
+ 1	− .4	.12	24.0	13.0	31.3
− 4	.0	.07	18.6	16.7	30.2
0	+ .6	.02	17.7	20.6	28.0
+25	+ .5	.02	20.5	14.6	35.1
+ 1	+1.1	.03	24.3	15.2	28.9
	Mean ± S.E.	.06 ± .013	21.4 ± 1.03	16.4 ± .91	30.7 ± .76
		.03 M malonate			
+ 5	+ .1	.20	29.4	14.7	29.3
− 1	+ .1	.43	21.0	11.6	34.8
+10	+ .2	.44	21.5	13.0	32.4
+ 6	− .1	.18	29.8	10.7	29.2
−10	+ .1	.12	26.2	14.5	31.6
− 6	+ .2	.13	21.9	18.4	29.4
− 7	.0	.18	24.2	17.9	28.2
0	+ .2	.19	26.7	15.1	26.9
− 6	+2.6	.44	17.4	16.9	30.0
− 4	− .3	.45	20.6	17.8	27.7
−27	+1.5	.59	10.3	21.7	30.3
− 2	− .6	.02			
− 6	+1.1	.14			
− 3	+ .1	.22			
− 3	+3.1	.28			
− 9	.0	.08			
− 5	+ .1	.06			
− 9	+ .3	.31	29.2	12.5	28.6
+22	+ .2	.15	28.6	12.9	28.5
	Mean ± S.E.	.243 ± .033	23.6 ± 1.6	15.2 ± .88	29.7 ± .6
		.06 M malonate			
−56	+1.2	.46	21.8	17.6	29.6
−78	−1.1	.14	28.3	12.4	26.8
−34	.0	.32	24.0	14.6	29.9
−69	−1.7	.36	29.0	9.9	30.2
−13	+1.5	.26	24.0	13.1	29.4
−56	+2.6	.22			
	Mean ± S.E.	.293 ± .016	25.5 ± 1.4	15.5 ± 1.6	29.2 ± .19

crease in rate. ECG changes were also observed such as prolongation of QT, electrical alternans and frequent multifocal premature ventricular systoles. Level of blood in the venous reservoir fell substantially owing to heart dilatation. The reservoir was not raised to restore venous inflow level for fear of intensifying the arrhythmias, so that the observed lack of significant rise in venous pressure, and the decrease in systemic output are of little value in assessing cardiac competence. Significantly, phosphorus values are unchanged, the phosphocreatine values, as in 0.03 M malonate experiments, are even slightly higher than in control experiments (3).

Discussion. One purpose of the present investigation is to compare the actions of malonate *in vitro* and *in vivo*. It is shown that concentration of malonate (0.03 M) which according to Krebs and Eggleston(1) and of Smyth(2) almost completely interrupts the citric acid cycle in muscle mince, has no effect on performance of heart-lung preparation or on phosphorus compounds of muscle, despite accumulation of succinate. Results obtained

by treating the heart-lung preparation with double dose of malonate are difficult to interpret. Succinate values are only slightly higher than with the single dose, and the high energy phosphate values are unchanged. There is thus no correlation between succinate accumulation and heart competence. The "failure" observed with the double dose was sudden, appearing before the whole dose was added, and was characterized by significant ECG changes. It may not be due to interruption of the Krebs cycle. It could well be due to hypertonicity of the serum, or binding of certain cations by malonate, or some unknown effect on myocardium. It has already been shown, for instance, that heart failure due to dinitrophenol cannot be attributed to its known action of uncoupling phosphorylation and oxidation(10). Other authors using different preparations have also noted an "anomalous" behavior of heart muscle to malonate(6,7,8).

Our experiments do not prove that succinate oxidation is not essential for cardiac function or that alternative pathways are functioning in malonate treated hearts. Malonate inhibition in our system may not be complete and succinate oxidation may not be the rate limiting step in the Krebs cycle. Further experimentation is necessary.

We have no explanation for variations in succinate accumulation after malonate. Preliminary experiments indicate that nutritional state of animals is of no significance.

Summary. Malonate in a concentration (0.03 M) which almost completely blocks the Krebs cycle in muscle mince, has no significant effect on performance of the isolated dog heart (heart-lung preparation) or on its high-energy phosphorus compounds. 0.06 M malonate produces also no change in the phosphorus compounds, there is even a slight increase in phosphocreatine. The double dose of malonate, however, produces a sudden cardiac dilatation before the full amount is administered. This is accompanied by a decrease in heart rate, ECG changes and arrhythmias. Significant increases in heart succinate with both doses constitute presumptive evidence that malonate diffuses into heart muscle but this accumulation of succinate shows variations and it cannot be correlated with cardiac performance.

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Anti-Infective Action of an Anabolic Steroid. (23876)

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Taubenhaus(1-4) and others showed that formation of granulation tissue in animals and response to infection were stimulated by testosterone; and this seemed to be related to anabolic rather than androgenic action of testosterone. Moreover, Sala and Baldratti(5) screened a series of steroids synthesized in this laboratory(6) and showed that in castrated rats some testosterone analogs possessed potent effect on weight of levator ani muscle and weak androgenic activity as measured by change in ventral prostate gland.

TABLE I. Effect on Nocardosis. 12 mice/exp.

Treatment	Dosage, mg/kg/day	Lethal time–50 (days)	95% confidence limits
Controls	—	3.1	2.2–4.2
4-chlorotestosterone	15	5.9	4.3–8.0*
Testosterone	15	4.0	3.2–4.9
Cortisone	25	1.9	1.4–2.6*
Cortisone + 4-chlorotestosterone	25 15	3.8	2.8–5.2†
Cortisone + testosterone	25 15	3.0	2.4–3.8†

* Significant difference between controls and the various treatments, $P < .05$.

† Significant difference between cortisone and the various treatments, $P < .05$.

4-chlorotestosterone was of particular interest in this respect because it also lacked corticoid, progestational and estrogenic activity and the thymolytic effect was greater than that induced by testosterone. In normal rats it neither caused testicular atrophy nor influenced spermatogenesis(7). As a result of these studies, experiments were undertaken to compare the effects of 4-chlorotestosterone with that of testosterone and cortisone on anaphylactic shock in guinea pigs and on experimental infections in mice and rabbits.

Method. For anaphylactic studies guinea pigs were sensitized by single subcutaneous injection of .01 to .02 ml horse serum. They were similarly injected daily with either 7.5, 15 or 30 mg/kg/day of 4-chlorotestosterone acetate for 10 days. At end of 2nd week all animals were challenged with 1 ml horse serum administered intravenously. Experimental nocardosis was produced in mice by intra-abdominal administration and in rabbits by intradermal administration of *Nocardia asteroides* suspension. Treatment, subcutaneously, was started immediately after infection was induced and was continued for 10 days thereafter. In another series of experiments mice were treated daily with the various agents 3 days prior to infection with intra-abdominally administered *Staphylococcus aureus* suspension and continued for 3 days thereafter.

Results. Table I shows that 4-chlorotestosterone effectively prolonged survival time of mice infected with *N. asteroides*. Testosterone was ineffective and cortisone hastened deaths. Although 4-chlorotestosterone or testosterone in combination with cortisone extended the survival time beyond that observed with cortisone alone, this effect was not significantly different from that obtained with untreated controls. Similarly, 4-chlorotestosterone doubled the survival time of mice infected with *S. aureus* (Table II) and was more effective in this respect than tetracycline.

In rabbits with cutaneous nocardosis 15 mg/kg/day 4-chlorotestosterone hastened healing of abscess by intensification of regenerative process and stimulation of granulation. 10 mg/kg/day cortisone, on the other hand, intensified the disease process and produced rapidly spreading purulent lesions. When 4-chlorotestosterone was administered together with cortisone the effect of the latter was prevented, healing was accelerated, necrosis was limited and proliferation of the surrounding tissue was stimulated.

4-chlorotestosterone in doses up to 30 mg/kg/day had no protective action in guinea pigs sensitized to horse serum. Actually, the mortality rate and tissue injury were markedly increased. The serum proteins, however, were not significantly affected.

Discussion. The non-specific body response to allergic or infective stimuli occurring in animals treated with 4-chlorotestosterone was significantly higher than that observed for controls. This non-specific stimulation was observed also in animals treated with cortisone; the proinfective action of cortisone, however, appeared to be nullified, without production of histologic changes. These conditions were particularly evident for *N. as-*

TABLE II. Effect on Staphylococcal Infection. 12 mice/exp.

Treatment	Dosage, mg/kg/day	Lethal time–50 (days)	50% confidence limits
Controls	—	5.3	2.2– 8.0
4-chlorotestosterone	15	10.5	6.6–16.6*
Tetracycline (Achromycin)	1	5.8	4.0– 8.4
Tetracycline + 4-chlorotestosterone	1 15	9.8	6.6–14.6*

* Significant difference between controls and treatment, $P < .05$.

teroides and *S. aureus* infected animals whose survival times were significantly prolonged. *In vitro,* however, 4-chlorotestosterone did not inhibit development of the infecting strains. According to these data the particular anti-infective action of 4-chlorotestosterone is not attributed to a direct chemotherapeutic effect but rather to a stimulating action of the steroid on organic reactive systems; particularly those inhibited by cortisone and conditioning the organic response to allergic or infective stimuli.

Summary. 4-chlorotestosterone is an anabolic steroid comparable to testosterone, but with less androgenic potential. It enhances the intensity of anaphylactic shock in guinea pigs, prolongs survival time of mice infected with *Nocardia asteroides,* or *Staphylococcus aureus,* hastens healing of cutaneous nocardosis in rabbits, and nullifies the proinfective action of cortisone.

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Comparison of Porphyrin Producing Enzyme Activities in Harderian Glands of Mice and Other Rodents.* (23877)

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It was observed that the Harderian glands of rats, hamsters, and most mice fluoresced red, while the Harderian glands of rabbits and guinea pigs did not(1-5). It had been assumed, and was later found, that the red fluorescent Harderian glands contained relatively greater quantities of porphyrin than those that did not fluoresce red. The enzymes for producing porphyrin must, however, be present in all Harderian glands because free protoporphyrin has been extracted from Harderian glands that were not red fluorescent. It was, therefore, of interest to determine the quantitative aspects of porphyrin producing enzymes in Harderian glands of various rodents. It was especially desirable to compare amounts of enzyme activity present in rat and hamster, where the glands are usually red fluorescent, with the amount of enzyme in Harderian glands in guinea pigs and rabbits, where these glands are usually not red fluorescent. In some rodents, where Harderian glands are white fluorescent, the porphyrin is present in such small quantities that its red fluorescence is masked by presence of blue-green fluorescent material. The blue-green and red fluorescence spectra are complimentary and the glands appear to be white fluorescent. In the rabbit, the Harderian gland consists of a dark and a white fluorescent portion. The white fluorescent portion contains by far the most porphyrin, but not nearly as much as the Harderian glands of rats and hamsters.

The results of these experiments on quantitative aspects of porphyrin producing enzymes in Harderian glands of rodents indicate that while these enzymes were present in rabbit and guinea pig Harderian glands, the enzyme activity in the rabbit gland was less than half that of the rat or hamster. In the guinea pig the enzyme activity was only 20%

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of that in the Harderian glands of rats and hamsters.

Materials and methods. The Harderian gland tissues used were obtained from commercially available male albino rats, rabbits, guinea pigs, and brown hamsters. The animals were housed in metal cages and given Purina Laboratory Chow and water without restrictions. Prior to removal of Harderian glands, the animals were anesthetized with ether and tissues dissected out in a cold room (6°C). The glands were removed as rapidly as possible, care being taken to exclude extraneous tissues. One hundred mg of Harderian gland tissue was weighed and placed in a mortar with small amount of coarse grinding sand and 2 cc of 50:50 buffer-saline solution. The gland tissue was homogenized with pestle and rinsed from the mortar with 3 cc of buffer-saline solution. The sand and coarse material were eliminated by centrifugation at 2500 rpm for 15 minutes. The supernatant material was used as the enzyme solution. The same procedure was followed to prepared enzyme solutions of brain, liver, and muscle. To each of 24 Klett tubes (6 for each tissue to be tested) 1.4 cc of 0.01 M δ-aminolevulinic acid in buffer-saline solution was added as the substrate. The buffer-saline solution consisted of equal volumes of isotonic sodium chloride solution and buffer solution pH 7.2 (0.029 molar potassium dihydrogen phosphate; 0.013 molar sodium tetraborate). The amounts of enzyme solution from each tissue to be tested were pipetted into the 6 tubes as follows: Tube No. 1—none (control); Tube 2—0.1 cc; Tube 3—0.2 cc; Tube 4—0.4 cc; Tube 5—0.8 cc; Tube 6—1.6 cc. Buffer-saline was added to each tube to bring volume to 3 cc. The tubes were then stoppered with cellophane covered corks and incubated at 37° for 24 hours. At the end of this period, 6.5 cc of concentrated HCl was added to each tube to stop the reaction and to clear the solution. This produced a 25% HCl solution. The tubes then stood overnight in cold room (6°C). A solution of hematoporphyrin hydrochloride in 25% HCl was prepared as standard to be used in a Klett-Summerson filter photometer to determine amounts of porphyrin formed. These experiments were repeated twice on 2 sets of animals.

TABLE I. Porphyrin Production by Aqueous Extracts of Harderian Gland and Other Rodent Tissue Homogenates.

		Enzyme extract conc. μg of porphyrin/3 cc of reaction mixture/24 hr				
Rodent	Tissue	0.1	0.2	0.4	0.8	1.6
Mouse†	Hard.	7.1	12.8	32.3	49.5	100.7
Rat	gland	6.6	11.5	29.4	48.7	100.0
Hamster		5.2	9.8	27.7	46.7	97.8
Rabbit (R)		.8	1.7	4.4	8.2	16.3
" (W)		2.4	5.5	14.5	19.3	48.3
Guinea pig		.9	1.9	4.9	8.3	18.8
Mouse†	Liver	5.5	9.5	28.5	34.6	73.4
Rat		5.2	9.0	28.7	34.2	71.0
Hamster		4.7	8.3	27.8	33.4	69.4
Rabbit		5.7	8.3	27.8	33.9	69.2
Guinea pig		5.7	8.0	26.1	32.4	69.8
Mouse†	Brain	.8	2.5	4.4	5.2	9.7
Rat		.5	2.4	4.0	5.1	8.8
Hamster		.1	2.0	3.6	5.2	8.7
Rabbit		.3	1.8	4.1	4.8	8.8
Guinea pig		.1	1.8	4.5	5.0	8.2
Mouse†	Muscle	1.8*	3.4*	6.7*	8.7*	16.1*
Rat		0	0	.4	1.2	3.1
Hamster		0	0	.3	.8	2.5
Rabbit		0	.1	.6	1.3	2.8
Guinea pig		0	.2	.8	1.7	2.9

R = postero-ventral lobe of Harderian gland.
W = antero-dorsal " " " " .

* Muscle plus bone and bone marrow.
† See reference (7).

Results. Variation in amount of porphyrin produced by different tissue enzyme preparations is shown in Table I. It may be seen that the amount of porphyrin produced by Harderian glands of different rodents did vary considerably. The amount of porphyrin produced in tubes containing extracts of the Harderian gland homogenates from the rat and hamster was far greater than that in rabbit and guinea pig. This corresponds to the relatively intense red fluorescence seen in rat and hamster and lack of appreciable red fluorescence in rabbit and guinea pig Harderian glands.

The Harderian gland of the rabbit consists of 2 parts; (W) the white antero-dorsal lobe and (R) the grey-reddish, postero-ventral lobe(6). The white (also white fluorescent) antero-dorsal lobe when tested alone possessed a greater amount of enzyme activity than did the grey-reddish postero-ventral lobe when tested by itself.

Liver, brain, and muscle extracts of the

same animals also exhibited a definite ability to convert δ-aminolevulinic acid to porphyrin. The porphyrin producing potentialities of liver tissue extract were, however, less than that of Harderian gland in rat and hamster, but more than in rabbit (both lobes) and guinea pig. The Harderian gland and liver enzyme extracts showed greater activity than brain and muscle in all instances.

In contrast to the observations on Harderian glands, enzyme activities of liver, brain, and muscle of all rodents tested were approximately the same when corresponding tissues were compared in the different rodents.

Discussion. It is of interest that porphyrin producing enzymes in the extracts of homogenates of liver, brain, and muscle did not vary appreciably in porphyrin producing enzyme activity in different rodents. In contrast, a great variation in enzyme activity of Harderian glands was found. The variation in amount of enzyme activity in the Harderian glands of various rodents parallels the degree of red fluorescence observed. This is related to the fact that there is more porphyrin in the rat and hamster Harderian gland than in rabbit and guinea pig. These observations, however, still give no clue as to the possible function of the porphyrin produced by Harderian glands. For example, it is not clear why rats and hamsters and mice should need a Harderian gland that produces more porphyrin than rabbit and guinea pig. An age study in mice revealed that there was a close parallelism between development of sex maturity and appearance and accumulation of porphyrin producing enzymes in the Harderian glands(8). It has been pointed out earlier that animals with relatively large amounts of porphyrin in the Harderian glands were quite susceptible to cancer(4,5). That is, mice, rats, and hamsters are quite susceptible, and other animals seem to be less susceptible. The hypothesis that there is a relationship between porphyrin metabolism and cancer susceptibility is still attractive, but no mechanism which would explain such a relationship has been discovered.

Summary and conclusions. 1. A quantitative study was made of porphyrin producing enzyme activities of Harderian glands, livers, brains, and muscles of mice, rats, hamsters, rabbits, and guinea pigs. 2. The tissues used were obtained from commercially available male albino rats, rabbits, guinea pigs, and brown hamsters, and strong A strain mice. 3. A buffer-saline solution or extract was made from a weighed quantity of each homogenized tissue mentioned. Measured amounts of these extracts were mixed with 0.01 M solution of δ-aminolevulinic acid and incubated at 37° for 24 hours. 4. Harderian gland extracts of mice and rats showed greatest amount of porphyrin producing enzyme activity, while the same tissue from the hamster was only slightly lower in enzyme activity. The 2 lobes of the Harderian gland of rabbit and guinea pig contained far less porphyrin producing enzyme. The white fluorescent antero-dorsal lobe of the rabbit contained considerably greater quantities of porphyrin producing enzymes than the grey-reddish postero-ventral lobe. 5. Liver, brain, and muscle extracts of the same rodent revealed amounts of porphyrin producing enzyme, uniform for tissue in various rodents and these values compared favorably with previous values determined for same tissues in the mouse. 6. Because of great variation in amounts of enzyme in various Harderian glands, the liver homogenate extract from rat and hamster contained less porphyrin enzyme than the Harderian gland. The reverse, however, was true in rabbit and guinea pig, for liver tissue contained more porphyrin producing enzyme than the Harderian glands of these rodents. It may be concluded that porphyrin produced by Harderian glands may have some function. This comparative study failed to give any clue as to what the function may be.

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Pharmacologic Characterization of Reserpine Responses in Rats Pre-Treated with Iproniazid.* (23878)

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Reserpine is known primarily for its hypotensive and sedative activities(1). It causes also liberation of pressor amines, notably epinephrine, norepinephrine and serotonin(2). Pressor activity consequent on release of these substances has been unmasked in anesthetized dogs and cats(3-5) by pre-treatment with iproniazid, a mono-amine oxidase inhibitor and attributed to impaired degradation of norepinephrine(4). In conscious mice, rats and rabbits, pre-treatment with iproniazid altered the response to reserpine from sedation to excitation(3,6); this change was attributed primarily to release of serotonin and its delayed inactivation(7,8). The present report concerns effects of reserpine on arterial pressure and behavior of anesthetized and conscious rats pre-treated with iproniazid and attempts to characterize the factors involved in these responses.

Methods. Experiments were done in male Sprague-Dawley rats weighing 175-255 g each. Except when indicated, anesthesia was induced with sodium amytal (9 mg/100 g body weight intraperitoneally) and vascular reflexes were inhibited with pentolinium bitartrate (5 mg solution in 20% polyvinylpyrrolidone subcutaneously); blood pressure was recorded following carotid cannulation (9). When given, iproniazid phosphate (25 mg intraperitoneally) was injected 18-24 hours before the test. Free reserpine dissolved in glycol vehicle was administered intraperitoneally in a dose of 5 mg/kg after arterial pressure had stabilized. Drugs used in evaluating the nature of responses were chlorpromazine hydrochloride (10 mg/kg intraperitoneally), lysergic diethylamide (to 250 μg/kg intravenously) and phenoxybenzamine hydrochloride (to 5 mg/kg intravenously). These were given 30-90 minutes before injection of reserpine. Parallel studies of symptomatic effects were done in conscious animals.

Results. 1. *Pressor effects of reserpine.* In rats treated with pentolinium but not with iproniazid, reserpine produced a small, transient initial decrease in arterial pressure followed by gradual rise to maximum of about 20 mm Hg above control levels; this pressor effect persisted for 90 minutes (Fig. 1A). In rats given both iproniazid and pentolinium, the response to reserpine consisted, as above, of initial small fall in pressure, followed by sharp rise which reached a maximum between 50 and 100 mm Hg within a half hour after injection (Fig. 1B) and remained at this level to 90 minutes. In rats given iproniazid but not pentolinium, reserpine was substantially as pressor as in rats given iproniazid and pentolinium; since post-iproniazid responses were presumably maximal, we could not demonstrate pressor sensitizing effect of the ganglion blocking agent.

2. *Effects of blocking agents on responses to reserpine.* While LSD had no definite effect, chlorpromazine inhibited but did not wholly prevent pressor activity of reserpine in iproniazid treated animals (Fig. 2A): under the same conditions, the same dose of chlorpromazine inhibited almost completely pressor effects of serotonin (1 μg). Phenoxybenzamine, an adrenergic blocking agent, reversed the effect of reserpine in iproniazid treated

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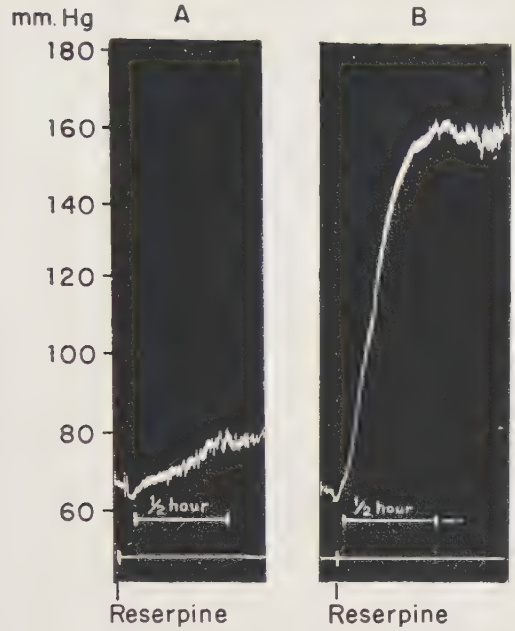


FIG. 1. Anesthetized, pentolinium treated rats. Pressor effects of reserpine in control (A) and in iproniazid treated (B) rats.

rats, so that it caused a prolonged depressor response (Fig. 2B); the maximum fall in pressure varied from 10 to 40 mm Hg. Animals given phenoxybenzamine but not iproniazid, also showed a depressor response, but this was small and corresponded to the small pressor response provoked by reserpine in rats not given phenoxybenzamine. In other experiments it was determined that this dose of phenoxybenzamine was sufficient to reverse responses to .2 μg of epinephrine and norepinephrine from pressor to depressor. As demonstrated in the dog(4,5) the main pressor agent released by reserpine in the rat seems to be norepinephrine; removal of adrenals, principal source of epinephrine, did not decrease pressor response to reserpine.

3. *Effects of "blocking agents" in conscious animals.* Blocking effects of phenoxybenzamine and chlorpromazine on behavioral responses to reserpine after treatment with iproniazid were studied in 3 groups of conscious rats. All animals were given iproniazid and reserpine in dosages noted above. Thirty minutes before administration of reserpine, one experimental group received phenoxybenzamine intravenously and the other, chlorpromazine intraperitoneally. As described by Chessin *et al.*(3) control animals responded to reserpine with an adrenergic reaction, demonstrating piloerection, exophthalmos, salivation, increased motor activity and excitability. This syndrome did not appear in the 2 groups given blocking agents; whatever symptoms appeared were attributable to specific actions of the blocking drugs, namely ptosis with phenoxybenzamine and depression with chlorpromazine.

Discussion. Our experiments extend previous observations in anesthetized cats and dogs (3-5) to the anesthetized rat. They indicate that the pressor effect caused by reserpine depends primarily on release of adrenergic amines, presumably norepinephrine and not serotonin, since LSD did not affect the response while chlorpromazine, a weak adrenergic blocker, and phenoxybenzamine a strong one, did. Further, during these experiments phenoxybenzamine, unlike chlorpromazine, did not affect the pressor response to serotonin in anesthetized rats. Although it has been shown in the rabbit that epinephrine is liberated from the adrenal medulla in response to reserpine (10) the present, like previous(5) experiments, in adrenalectomized animals, indicate that its role is negligible. Similarly the adrenergic response to reserpine in iproniazid-treated rats is, as the descriptive term implies, largely attributable to release of pressor catecholamines. This view is supported by blocking effect of phenoxybenzamine on pressor and central nervous effects of reserpine and

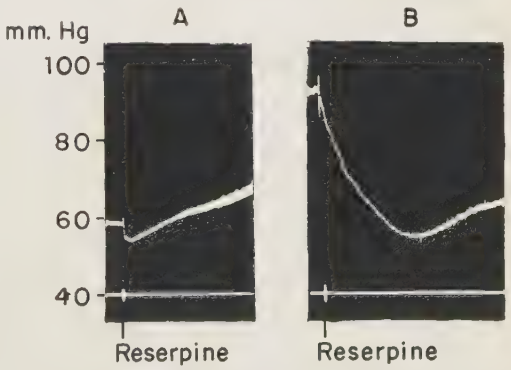


FIG. 2. Anesthetized rats pre-treated with iproniazid and pentolinium. Effect of chlorpromazine (A) and of phenoxybenzamine (B) on the response to reserpine.

by lack of effect of LSD, since in dosage used, LSD has been found to inhibit the effect of injected(11) and of infused(12) serotonin on arterial pressure of anesthetized rats. Further confirmation is found in the fact that in rats pre-treated with iproniazid but not given pentolinium, reserpine was consistently pressor while serotonin was depressor. Clinical corroboration is found in the response to phentolamine of hypertensive patients under treatment with rauwolfia drugs whose response is commonly depressor(13); it seems likely that this reflects a state of underlying adrenergic excitation which in a degree mimics the status of patients with functioning pheochromocytoma.

Summary. In anesthetized rats pretreated with iproniazid, injected reserpine elicits a marked pressor response which from observations on effects of phenoxybenzamine, chlorpromazine and LSD seems primarily attributable to release of adrenergic amines, presumably norepinephrine. Experiments in conscious rats indicate that their response to reserpine is adrenergic. The data support conclusions reached by others in other species but do not support the view that serotonin plays a large role.

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Biliary and Fecal Vit. B_{12} Excretion in Man. An Isotope Study.* (23879)

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In spite of considerable progress in Vit. B_{12} research, it is still not known what amount normal humans have to absorb in order to maintain health; likewise, the optimum maintenance dose for pernicious anemia patients is unknown. One way to elucidate these questions is to study quantitatively the excretion of Vit. B_{12}. As judged from the minimum maintenance dose for pernicious anemia patients, normal humans seem to absorb at least one microgram *pro die* under physiological circumstances(1). When pure Vit. B_{12} is given *per os,* the upper limit of absorption lies well above this figure(2,3). On the other hand,

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normal humans excrete less than one microgram in the urine per day(4). This indicates that the vitamin is either broken down in the body or at least is excreted in urine as a microbiologically inactive compound, or, alternatively, is excreted mainly by routes other than the urine. Previous studies on animals(5,6) demonstrate the importance of the biliary and fecal routes in the excretion of "endogenous" (absorbed) B_{12}. Indications of the significance of these routes in man were obtained with the finding of high microbiological B_{12} activity in human bile(5,6). Also, as judged from a few observations reported in the literature(7,8,9), it seems that in man parenterally administered radiovitamin B_{12} appears in the bile and feces. However, in no case has this excretion been subject to a systematic study. Because of the relatively large doses administered, the short observation times, and the change in excretion pattern of radiovitamin during the first days after injection (Fig. 1), no definite conclusion can be drawn as regards the physiological excretion routes of Vit. B_{12}.

In the present study it was attempted to study systematically the biliary and fecal B_{12} excretion by administering parenterally minimal doses of radiovitamin B_{12} and by subsequently following for long periods its elimination with the bile and feces. It was felt that such an investigation could not be restricted to the fecal route alone, since the extensive synthesis and possible breakdown(10) of B_{12} taking place in the intestinal contents make it impossible to draw any conclusions as to whether the fecal radioactivity is derived from excreted Vit. B_{12}.

Material and methods. A single 0.5 μg dose of Co^{56} labelled Vit. B_{12} with a specific activity of approximately 2 mc/mg (the authors are greatly indebted to Dr. E. Lester Smith, Glaxo Ltd., England, for the generous gift of $Co^{56}B_{12}$) was injected intramuscularly to the following subjects: a) 5 healthy young adult females, whose feces were subsequently collected in one day portions for 26-29 days; 24-hour urine samples were collected the first 2 days after injection and thereafter at irregular intervals; b) 5 patients with a postoperative T-tube drain in the common bile duct and without clinical signs of disordered B_{12} metabolism and whose drain bile was collected in one day portions until the drainage was discontinued; c) 2 healthy young adult males, who were subjected to duodenal lavage 3 times during the 1st to 40th day after the injection. The duodenal lavage was performed in the usual manner by passing down the tube during X-ray fluoroscopy and by injecting magnesium sulfate through the tube. Before counting, the feces samples were liquefied by nitric acid digestion, and the bile and urine samples were concentrated by evaporation. In counting, scintillation technic was used. Since it is not possible, even by drainage, to collect the daily bile secretion quantitatively it was attempted to calculate the daily biliary Co^{56}-B_{12} excretion in the following manner: the bilirubin content of the bile samples was determined, and the radioactivity found was corrected assuming that the daily bilirubin excretion was 270 mg.†

Results. The excretion pattern changing markedly after the first few days following injection, the data have been grouped into an *early* (1st-4th day) and a *late* (4th-30th day) group (Fig. 1).

Urinary excretion. Even with the very small amounts of B_{12} administered in this study, considerable amounts of radioactivity are eliminated by this route during the first 2 days after injection. During the early period, more Vit. B_{12} is excreted by this route than by any other (Table I, Fig. 1). On the other hand, during the late period the urine contained less radioactivity than the feces. In contrast to the feces samples, which all contained demonstrable radioactivity, in some late urines no activity could be detected.

Fecal excretion. Although the values observed early after the injection are distinctly higher than later on, this effect is much less marked than in the urine (Table I, Fig. 1). During the late period, the mean 24-hour excretion of radioactivity varies relatively little

† This was calculated as follows: Normal total body hemoglobin is approx. 750 μg(20), corresponding to 29.0 g of heme. Calculating with a breakdown of 1/120/day, this corresponds to excretion of 230 mg of bilirubin. To this are added 40 mg from non-hemoglobin porphyrins(21,22).

TABLE I. Daily Excretion of Radioactivity, Calculated as B_{12}, after Intramuscular Injection of 0.5 μg Co^{56}-B_{12}.

			mμg/day	Range	% excreted of total B_{12}-Co^{56} in body	No. patients	No. of 24-hr collections analyzed
Early (1st-4th day)	Bile	Observed	4.8 ± 1.4†	.6–10.5		7	18
		Calculated	13.2 ± 3.9	2.6–29.1		7	18
	Feces		4.6 ± 2.0	1.5– 8.6		5	15
	Urine*		25.8 ± 2.4	12.9–32.9		5	10
Late (4th-30th day)	Bile	Observed	.7 ± .2	.4– 1.6		7	19
		Calculated	5.1 ± 1.3	1.4–11.5	1.1 ± .3	7	19
	Feces		1.4 ± .1	1.1– 1.7	.3 ± .02	5	123
	Urine		.1 ± .09	.0– .5	.02± .02	5	10

* Urine collected for 2 days only.

† Mean ± S.E.

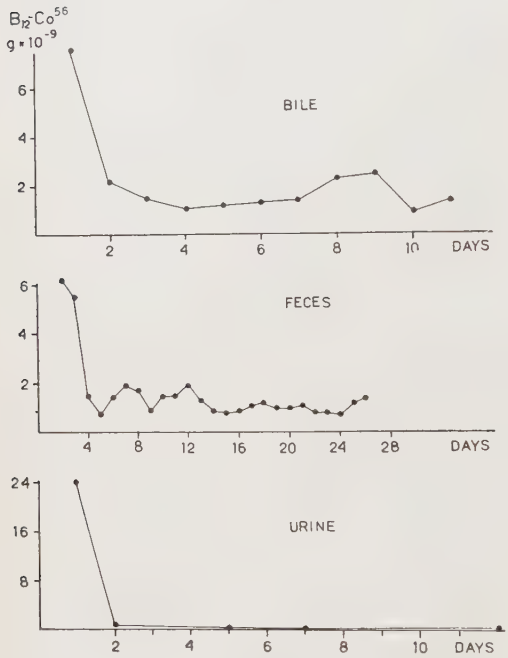


FIG. 1. Difference between *early* (1-4th day) and *late* (4th-30th day) period after parenteral administration of vit. B_{12}-Co^{56} as regards the pattern of radiovitamin excretion in 3 cases. Regarding excretion in bile and urine, those cases were chosen in which the largest number of samples were obtained. As regards fecal excretion, the case was chosen at random. There was considerable day-to-day variation, probably explained by differences in frequency of defecation and quantity of stools passed on different days. The curve represents successive mean values.

in different subjects. No feces samples were found to be nonradioactive. The average amount of radioactivity excreted by this route per day during the late period (calculated as Co^{56}-B_{12}) corresponds to 0.3% of the radioactivity present in the body (injected amount *minus* that excreted during the early period).

Biliary excretion. The early values are distinctly higher here also. The mean late excretion value is 5.1 mμg/day, corresponding to 1.1% of the amount of radiovitamin B_{12} present in the body after the early period (Table I).

The calculated corresponding excretions of "endogenous" vitamin are seen in Table II.

Discussion. The question naturally arises, whether the behavior of the injected radiovitamin is representative of the fate of the nonlabelled vitamin normally contained in the body.

The results of other workers(11-14) who have studied the distribution of radioactivity in different organs after oral or parenteral administration of radiovitamin B_{12}, indicate a

TABLE II. Daily Excretion of Endogenous Vitamin B_{12}. The calculations have been based on the assumption that the injected radiovitamin mixes with the body stores.

	Mean ± S.E.	Range
	μg	
Total amt of B_{12} in body*	3,900	790–11,100
Corresponding daily excretion of endogenous B_{12}		
Bile	43 ± 12	9 –122
Feces	12 ± 1	2 – 33
Urine	.8 ± .8	.2– 3

* Obtained by adding microbiological B_{12} contents of liver, kidneys, heart, spleen, brain, and muscles in 4-22 autopsies (varying for different organs)(15,16). The corresponding excretion of endogenous B_{12} is calculated using percentages given in Table I. Stand. errors are based only on isotope studies and do not include those of microbiological assays.

relatively good mixing of the introduced isotope with the body B_{12} pool. The distribution of radioactivity closely resembles the natural distribution of microbiological B_{12} activity (15,16).

Partly because this study deals with a human material it is difficult to establish definitely the time required for the complete mixing of the isotope among the body B_{12}-pool. In the present study the fourth day was chosen as the limit, because by this time the excretion pattern had undergone a marked change from the dominantly urinary excretion pattern into one characterized by a steady fecal output of radioactivity. In addition, other investigators(13,14) have found that by this time the distribution of parenterally injected radiovit. B_{12} has assumed the "final" pattern with maximal activity over the liver area. Although the percentage excretion of radioactivity does not seem to be much affected by the fixing of the limit to a few days later than the 4th day after the injection, the question on the mixing of injected radiovit. B_{12} has to be subjected to further study. Until such studies are concluded, the data given on the excretion of endogenous Vit. B_{12} should be interpreted with caution.

Irrespective of whether the mixing within the period of time studied in the present paper is absolutely complete, the conclusion seems well founded that in man Vit. B_{12}, or its cobalt containing breakdown product, is excreted mainly by the fecal route. The source of the fecal material may well be the bile, which was found to have relatively high radioactivity and which is rich in microbiological B_{12} activity(5,6). Our data indicate that at least two-thirds of the biliary radioactivity is reabsorbed from the intestine, and thus that Vit. B_{12} is subject to an enterohepatic circulation as are other components of the bile. It is possible that this reabsorption is aided by the gastric intrinsic factor and/or the Vit. B_{12} binding substance found to occur in bile(5,6). An impaired intestinal reabsorption may be a factor in the pathogenesis of certain B_{12} deficiency states. Preliminary results with simultaneous collection of bile and feces in the same subjects seem to confirm the concept of an intestinal reabsorption of Vit. B_{12}(17).

That absorbed Vit. B_{12} is excreted by the bile-feces route probably explains the fact that in performing fecal excretion tests, some radioactivity can be detected in the stools for a long time after the administration of the oral dose. Biliary excretion with subsequent isotope dilution in the intestine may explain the observed inhibition of radiovitamin B_{12} absorption by parenteral administration of nonlabeled B_{12}(18).

Provided the introduced radiovitamin is uniformly mixed with the nonlabeled vitamin contained in the body, it should be possible to calculate from the present data the corresponding excretion of endogenous B_{12} if the size of the B_{12} pool be known. Thus far tentative calculations have been performed, in which the size of the pool has been estimated on the basis of microbiological assays performed by other authors(15,16) on autopsy material. The results are listed in Table II. Even allowing for considerable errors, the data seem to indicate that the daily loss of Vit. B_{12} may amount to several micrograms, and thus the requirement of B_{12} may be larger than hitherto assumed(1). The order of the magnitude of the daily loss of Vit. B_{12} receives some support in recent investigations on the hepatic storage of radiovit. B_{12}(11,19), indicating a loss of several micrograms of radiovitamin per day.

Summary. It was attempted to study Vit. B_{12} excretion in man by investigating the elimination of small doses of parenterally administered radiovitamin Co^{56}-B_{12}. Relatively large amounts of radioactivity were excreted in the bile, feces and urine shortly after injection. After this period, the excretion pattern changed, and radioactivity of relatively constant value was excreted with the bile and feces, indicating that these are the main excretion routes in man, whereas the urine samples exhibited low radioactivity. The fecal radioactivity was only about one-third of the calculated biliary excretion, which indicates intestinal reabsorption of biliary B_{12}. If it were assumed, that the radiovitamin mixes uniformly with the body stores of Vit. B_{12}, it could be estimated that normal subjects may lose considerable amounts of B_{12} per day, and consequently, the requirement of

B_{12} could be larger than hitherto suspected. The findings may have therapeutical implications.

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Effects of Δ^4-Cholestenone in Animals and in Man. (23880)

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Feeding of large amounts of Δ^4-cholestenone* will lower the levels of serum cholesterol in rats(1,2), dogs and chickens(3). This effect on serum cholesterol may be related to suppression of endogenous cholesterol biosynthesis by cholestenone, shown first by Tomkins, Sheppard and Chaikoff in acute experiments(4) and confirmed in this laboratory in chronic feeding experiments(1). However, high dose levels (1 g/kg/day) of cholestenone are highly toxic to rats, causing sharp arrest of normal growth rate and accumulation in tissues of dihydrocholesterol, a major product of cholestenone metabolism(5-7). The other outstanding effect of chronic feeding of cholestenone at high dose levels is a remarkable hypertrophy of the adrenal gland to 7 times normal weight(1). This hypertrophy is associated with sharply reduced rate of secretion of corticosteroids—both corticosterone and aldosterone(8). The present studies were carried out to evaluate general toxicity of cholestenone in different species; to study absorption and metabolic fate of the compound, particularly degree of conversion to dihydrocholesterol; and to further explore adrenal changes induced by large doses.

Methods. Male Osborne-Mendel rats were maintained on basic diet of following percentage composition: casein, 34.5; sucrose, 31.0; corn starch, 21.5; cottonseed oil, 6.5; salt mixture(9), 5; cod liver oil, 1; choline, 0.1 and supplements of vitamins. Cholestenone,† dihydrocholesterol and cholesterol were

* Referred to subsequently simply as cholestenone.

† We are indebted to Dr. Preston L. Perlman of Schering Corp. for supplying Δ^4-cholestenone and dihydrocholesterol.

incorporated into basic diet at various levels and the diet fed *ad lib.* Rabbits and guinea pigs were fed standard laboratory chow (B. and B. Small Animal Laboratory Diets). Sterols were added to these diets by blending appropriate amount of ether solution of sterol with weighed amount of ground pellets and then allowing solvent to evaporate. *Lipid analyses.* Published procedures were used for determination of serum cholesterol(10) lipid phosphorus(11) and total lipids(12). Tissue lipids were extracted by homogenizing the tissue in Waring blendor for 3 minutes with 1:1 acetone-ethanol and allowing homogenate to stand overnight at room temperature. Aliquots of extract were analyzed as in the case of serum lipids. Dihydrocholesterol was determined indirectly. Acetone-ethanol extracts of serum were saponified and total cholesterol was determined on one aliquot by Liebermann-Burchard color reaction(13). The digitonin-precipitable sterols were recovered from a second aliquot. The digitonide was washed 3 times with 50% ethanol, once with ether: acetone (2:1), and finally with ether, then dried to constant weight. The contribution of cholesterol, calculated from Liebermann-Burchard color yield, was subtracted and the difference attributed to digitonide of dihydrocholesterol. On several samples, the digitonides were cleaved with pyridine and the recovered sterols were dried to constant weight. The percentage of cholesterol therein as determined by Liebermann-Burchard reaction was in very good agreement with that obtained by the first method. These indirect methods would include any 3-OH sterol yielding little or no color under conditions of Liebermann-Burchard reaction but forming a digitonide with solubility like that of cholesterol. For this reason we have used single quotes throughout to indicate 'dihydrocholesterol' determined by these methods. While these determinations are not specific, the large amount of 'dihydrocholesterol' found in serum and tissues of experimental animals described below is most likely correctly identified as such(14). *Radiochemicals and radioassay.* Cholesterol-4-C^{14} was purchased from Beta Laboratories; cholestenone-4-C^{14} from Tracerlab. Liquid scintillation counting was used for radioassay of lipid fractions (Packard Tri-Carb Scintillation Spectrometer). Samples were taken to dryness in the counting bottle and 15 ml of an 0.6% solution of diphenyloxazole in toluene were added. When necessary, as in counting of highly colored fecal lipid extracts, a correction for quenching was made after adding known amount of palmitic acid-1-C^{14} to each vial and recounting. *Other analyses.* Ascorbic acid was determined by titration of cold 5% trichloroacetic acid (TCA) extracts of tissues with 2, 6-dichloroindophenol(15). In some instances the method of Roe(16) was also applied but in no case was more than 5% of ascorbic acid present in oxidized form. Protein nitrogen was determined by micro-Kjeldahl analysis of TCA-insoluble tissue residues after extraction of nucleic acids with hot TCA and extraction of lipids with alcohol-ether(1:1) and ether.

Results. General toxicity. Fed at a level of 1% by weight in the basic diet, cholestenone is highly toxic to rats, as previously reported(1). Appetite fails and growth curve quickly falls below that of rats on control diet. Since cholestenone was fed as constant percentage in diet, the daily intake of the compound varied with size of rat, ranging from about 1 g/kg in 100 g rats to about 700 mg/kg in 300 g rats that had been on the diet for 5 or 6 weeks. Growth was virtually arrested by the fifth week. Animals became lethargic, the coat became scruffy and there was crusting and discharge about the nose. There was a 20 to 30% mortality in 8 weeks. It was shown in paired feeding experiments that rats receiving control diet in amounts equal to that ingested by rats on cholestenone, had a growth curve not differing significantly from that of the latter (Table I). It was concluded that the principal effect of cholestenone on growth was due to decreased food intake, not to changes in absorption or energy metabolism.

Effects of cholestenone on adrenal gland. The most striking finding in rats given cholestenone at the 1% level was a remarkable hypertrophy of the adrenal glands(1). Histologic examination showed that hypertrophy was limited to the cortex. After 8 weeks on the drug the net weight of combined glands was 5 to 7 times that of glands from control

TABLE I. Adrenal Effects of Δ^4-cholestenone in Paired Feeding Study.*

Rat	Body wt (g) Initial	Body wt (g) Final	Combined adrenal wt (mg)	Adrenal ascorbic acid conc. (mg/g)	Adrenal cholesterol conc. (mg/g)	Relative thymus wt (mg/100 g)
Control A	112	185	40.6	5.88	57.4	190
Exp. A	113	185	202.6	.90	5.5	242
Control B	108	219	31.9	5.34	60.6	183
Exp. B	108	215	85.6	1.86		158
Control C	105	189	26.1	6.00	39.5	183
Exp. C	109	189	68.6	2.34	17.4	217

* Experimental rats were fed a 1% Δ^4-cholestenone diet *ad lib* for 4 wk; control rats were fed the basic diet in amounts equal to those consumed by the paired experimental rats.

rats of same age. Little or no increase in gland size occurred during the first 18 days on the diet but after 36 days there was a 3-fold increase. This hypertrophy is readily reversed by removing cholestenone from the diet. After 6 weeks on the drug, protein nitrogen/g wet weight was decreased by only about 20% while total mass had increased more than 3-fold. There was a definite increase in neutral fat, but the most impressive change was the marked fall in cholesterol concentration from normal value about 60 mg/g, to less than 10 mg/g. Phospholipid concentration did not change significantly. Another striking finding was an early fall in ascorbic acid concentration. After 18 days on cholestenone, although little or no adrenal hypertrophy had yet developed, there was a 38% fall in ascorbic acid concentration. After 36 days there was 85% decrease in ascorbic acid concentration. Again total quantities and concentrations of cholesterol in glands of treated animals were sharply depressed. Results from paired feeding study (Table I) indicate that this was not due to malnutrition.

Adrenal function in cholestenone-fed rats. As discussed previously(1), the known inhibitory effect of cholestenone on cholesterol biosynthesis suggested that the observed adrenal hypertrophy might be related to a similar block in adrenal steroid biosynthesis or to deficiency of new cholesterol to serve as steroid precursor. Findings of low cholesterol concentration and low ascorbic acid concentration would be compatible with such a postulate. Absence of thymic involution with marked adrenal hypertrophy (Table I) also suggested hypofunction at least/unit mass of gland.

Direct demonstration that adrenocorticosteroid secretion is in fact depressed was reported recently by Fredrickson, Peterson and Steinberg(8). Analyses were carried out on adrenal vein blood of pair fed rats on control diets and on 1% cholestenone diet. Output of corticosterone, the primary adrenal steroid in the rat, was reduced to 15% of control values after 6 weeks on cholestenone. Expressed as output/unit weight of adrenal gland, the rate of corticosterone secretion was only 3% of control values. Attempts to reverse the effects of cholestenone on growth by daily injections of cortisone (0.2 mg daily) were unsuccessful. Even when, in a second study, steroids were started at same time as cholestenone diet and given in larger doses (1 mg daily), growth rate was not improved compared to that of animals receiving no steroid. Corticosterone was no more effective than cortisone. Adrenal hypertrophy on the other hand, was completely or almost completely prevented, thus confirming the importance of pituitary stimulation in development of adrenal hypertrophy. Failure of exogenous steroid to permit normal growth rate suggests that at these high dosages cholestenone has toxic effects over and above those on adrenal glands.

Partial protective effect of dietary cholesterol. Our basic diet was virtually cholesterol-free. It was necessary to consider the possibility that inhibition of endogenous cholesterol synthesis by cholestenone was inducing a relative sterol 'deficiency'. Results of a feeding experiment to determine whether or not simultaneous feeding of cholesterol would protect against the toxic effects of cholestenone are shown in Table II. In terms of growth rate there was a decided protective ef-

TABLE II. Partial Protective Effect of Dietary Cholesterol against Toxic Effects of Δ^4-cholestenone Diet.†

	1% Δ^4-cholestenone alone	1% Δ^4-cholestenone + 1% cholesterol	1% cholesterol alone
Initial body wt, g	113 ± 12	116 ± 14	114 ± 10
Final body wt, g	187 ± 47	296 ± 35	307 ± 56
Serum cholesterol, mg %	43.4 ± 7.3	84.1 ± 13.9	80.2 ± 16.5
Combined adrenal wt, mg	136*	89*	36*

* Mean wt/pair of pooled glands.

† 10 rats in each group.

fect. There was a marked fall in serum cholesterol level of animals receiving cholestenone alone but none in animals receiving cholestenone plus cholesterol. Rats on cholestenone alone showed almost a 4-fold hypertrophy of adrenal glands; those receiving both compounds also had hypertrophied glands, but the degree of enlargement was again somewhat less than that in rats not receiving cholesterol. From these results it appears that simultaneous feeding of cholesterol does indeed partially ameliorate, although it does not completely prevent toxic effects of cholestenone.

Absorption and distribution of Δ^4-cholestenone-4-C^{14}. Two rats weighing approximately 250 g were given by stomach tube cholestenone-4-C^{14} dissolved in Wesson oil. Urine and feces were collected for 24 hours, then the animals were killed. Intestinal contents were pooled with feces and total lipids were extracted into acetone-ethanol (1:1) and counted. As shown in Table III, over 70% of administered dose was absorbed in 24 hours. Simultaneous administration of cholesterol did not interfere with absorption of labeled cholestenone (Table III). Less than 0.01% of administered radioactivity appeared in the urine. To determine organ distribution, a larger dose of cholestenone-4-C^{14} was given by stomach tube to a 210 g rat and the animal killed 20 hours later. Results are summarized in Table IV. Total radioactivity in the non-saponifiable lipid fraction of each organ was determined (cholestenone-C^{14} plus any labeled non-saponifiable products). Highest concentrations of radioactivity were found in liver and intestine. Other tissues contained relatively small amounts of radioactivity with the exception of serum and adrenal glands. In relation to organ weight the concentration in the latter was actually 10 times higher than that in liver. This relative concentration of cholestenone and its metabolites in the adrenal gland is of particular interest in view of the profound effects of the compound on adrenals discussed above.

The feces contained, besides the 5.4 x 10^6 cpm in non-saponifiable lipid, an additional 6 x 10^6 cpm in saponifiable form. This acid fraction most probably represents the modified bile acids shown by Harold, Abraham and Chaikoff to be end-products of cholestenone metabolism in the rat(7). Almost certainly the material in this fraction has been absorbed and subsequently re-excreted into the intestine. If it is included in the "absorbed" fraction in computing a radioactivity balance, 63% of administered dose was absorbed in experiment shown in Table IV. The value of 70% absorption indicated in Table III should, for similar reasons, be considered a minimum value.

Conversion of Δ^4-cholestenone to 'dihydrocholesterol'. That cholestenone is effectively reduced to dihydrocholesterol by rats was clearly shown by isotopic studies of Anker and Bloch(5) and amply confirmed by Stokes, *et al.*(6) and by Harold *et al.*(7). Little or no conversion to cholesterol itself could be demonstrated(5-7). In previous studies on rats receiving 1% cholestenone it was shown that in chronic feeding experiments 'dihydro-

TABLE III. Intestinal Absorption of Δ^4-cholestenone-4-C^{14} in the Rat.

Dose*			
Δ^4-cholestenone-4-C^{14} (mg)	(cpm)	Cholesterol (mg)	Radioactivity absorbed in 24 hr (%)
9.6	4.05 × 10^6	0	72
9.4	3.97 "	0	76
9.4	3.94 "	9.4	73
8.8	3.69 "	8.8	79

* Administered by stomach tube in 1.5 ml Wesson oil to rats weighing between 230 and 280 g.

TABLE IV. Distribution of Radioactivity 20 Hr after Oral Administration of Δ^4-cholestenone-4-C^{14}.*

Tissue	Total radioactivity (cpm)† × 1000
Liver	1,100
Intestine (washed)	505
Serum	280
Adrenals	176
Lungs	68
Spleen	44
Kidney	27
Heart	14
Brain	4
Remaining carcass	1,020‡
Feces§	5,400

* 60 mg (22.5 μc) Δ^4-cholestenone in 4 ml Wesson oil given by stomach tube 20 hr prior to sacrifice.

† Total nonsaponifiable lipid fraction counted in Packard Tri-carb liquid scintillation counter.

‡ 93% of this radioactivity could be precipitated with digitonin.

§ Total 20 hr fecal output pooled with intestinal content at time of sacrifice.

cholesterol' accumulates in liver and other tissues(1). The total non-saponifiable lipid in livers of animals receiving the compound for 8 weeks at 1% level, was more than doubled, while cholesterol content was not appreciably altered. We have now shown that there is an even greater relative accumulation of dihydrocholesterol in the adrenal gland. Here cholesterol concentration falls sharply (Table I) but *total* digitonin-precipitable sterol concentration actually rises somewhat (88 mg/g). Over 85% of this material is 'dihydrocholesterol'. The extent to which cholestenone is metabolized to dihydrocholesterol or to related 3-β-hydroxy forms is shown by studies of non-saponifiable carcass lipid after a single dose of cholestenone-4-C^{14}. As indicated in Table IV, 93% of non-saponifiable radioactivity was precipitable with digitonin, indicating that almost all of this material has been converted to a 3-β-hydroxy form.

Accumulation of 'dihydrocholesterol' in serum of rats is shown in Table V. The serum of animals on control diets showed a small amount of 'dihydrocholesterol' (3-6% of total sterol). After 40 weeks on low dose level of cholestenone (0.1%) the average level of 'dihydrocholesterol' was 31 mg%, accounting for 30% of total digitonin-precipitable sterol. Although serum cholesterol level was decreased in these animals, *total* serum sterol remained virtually unchanged. At the high dose level (1%) the 'dihydrocholesterol' concentration after only 7 weeks had reached 62 mg %, accounting for over 70% of total digitonin-precipitable sterol in serum. In this case total sterol level was actually *increased* even though cholesterol level was depressed more than 50%.

Effect of dihydrocholesterol feedings. In view of considerable amounts of dihydrocholesterol in serum and liver of animals receiving large doses of cholestenone it was necessary to consider the possibility that the observed toxic effects, in particular that upon the adrenal, might be due not to cholestenone itself but rather to this metabolic derivative. Dihydrocholesterol was fed to rats at level of 1% in diet. In marked contrast to results obtained with equal dosages of cholestenone there was little or no impairment of growth in an 8-week period. On the other hand, there was an almost 100% increase in adrenal weight and some fall in serum cholesterol (controls 54.4 mg%; dihydrocholesterol-fed, 44.6 mg %).

The fact that dihydrocholesterol was much less toxic in rats than cholestenone does not necessarily rule out the possibility, discussed above, that the former compound may be directly responsible for some of the toxic effects. Cholestenone appears to be better absorbed than dihydrocholesterol and, after conversion, may give rise to higher body concentrations of dihydrocholesterol than are

TABLE V. 'Dihydrocholesterol' in Pooled Sera from Rats on Δ^4-cholestenone Diets.

Diet	Weeks on diet	Serum cholesterol* (mg %)	Serum 'dihydrocholesterol'† (mg %)
Control (9)‡	40	95	6
.1% Δ^4-cholestenone (9)	40	74	31
Control (17)	7	55	2
1.0% Δ^4-cholestenone (37)	7	24	62

* Liebermann-Burchard reactive sterol.

† Sterol precipitated by digitonin but not Liebermann-Burchard reactive.

‡ No. of animals in each group shown in parentheses.

reached by feeding the compound itself.

Results at low dose levels. In contrast to results at high dose levels, drug levels up to 0.1% in the diet (70-100 mg/kg/day) did not impair appetite or limit growth. Rats have been maintained on this level of cholestenone in the diet for over a year without impairment of growth or other gross evidence of toxicity. Furthermore, cholestenone at 0.1% level caused no increase in adrenal weight in 7 weeks. After 40 weeks at this dose level there was approximately 30% increase in adrenal weight, but this was mostly due to lipid accumulation.

Clinical studies. It was of interest to study effects of cholestenone on patients with hypercholesterolemia, since abnormally high initial levels of circulating sterol might lead to a response different from that observed in normocholesterolemic experimental animals. Several patients with essential hypercholesterolemia were given 2.5 to 5.0 g cholestenone daily by mouth. This dosage corresponds to that given to rats receiving 0.1% cholestenone added to basic diet (60-80 mg/kg/day). The drug was well tolerated and there were no clinical signs or symptoms of toxicity. In view of the toxic effects of high dose levels in experimental animals (600-800 mg/kg/day) careful checks were made of hepatic, renal and adrenal function. None of the measured parameters changed with one exception. After several weeks there was a decided elevation in bromsulfalein retention test, up to 26%. None of the other measures of liver function (cephalin flocculation, thymol turbidity, prothrombin time, serum bilirubin and total serum proteins) were affected. The elevation of bromsulfalein retention in these patients was unexpected since at this dosage level in rats there was no accumulation of lipid in the liver and histologically no signs of hepatic damage. Clinical studies were interrupted because of this finding.

The effect on serum sterols in patients was also quite different from that seen in experimental animals on comparable doses. As shown in Table VI, patient C.B., an untreated hypercholesterolemic, had a small but significant amount of 'dihydrocholesterol' in her serum. Patients receiving 2.5 to 5.0 g cholestenone daily developed very high levels of dihydrocholesterol in serum, in one case actually exceeding the level of serum cholesterol. Levels of serum cholesterol did not change significantly. These results are in contrast with results in rats receiving equivalent dosage for 40 weeks, when cholesterol level fell significantly and total sterol level in serum did not rise.

TABLE VI. Serum 'Dihydrocholesterol' Concentrations in Hypercholesterolemic Patients Receiving Δ^4-cholestenone.

Patient	Dosage	Serum cholesterol* (mg %)	Serum 'dihydrocholesterol'† (mg %)
C.B.	None	509	21
F.G.	5 g/day for 46 days	475	549
	After 35 days off drug	365	70
J.K.	2.5 g/day for 32 days and 5 g/day for 70 days	278	212

* Liebermann-Burchard reactive sterol.
† Sterol precipitated by digitonin but not Liebermann-Burchard reactive.

Discussion. The present studies have shown that at low dose levels (70-100 mg/kg/day) Δ^4-cholestenone is well tolerated by rats for a year. Serum cholesterol level is decreased but total serum sterol level is not lowered since there is at the same time an accumulation of dihydrocholesterol. Similar dose levels in man lead to a much more striking accumulation of dihydrocholesterol in serum, in some cases doubling total sterol concentration. The elevation of bromsulfalein retention in patients on Δ^4-cholestenone suggests dihydrocholesterol accumulation in the liver also, an effect observed in rats only at much higher dose levels. Chapman, Nichols and Chaikoff have indicated similar results in birds(17). These findings, particularly in view of the possible atherogenic properties of dihydrocholesterol(18,19), appear to rule out use of Δ^4-cholestenone itself in therapy of hypercholesterolemia. Compounds with related structure but which cannot be converted to dihydrocholesterol deserve study.

At high dose levels (700-1000 mg/kg/day) Δ^4-cholestenone is highly toxic to rats. The unexpected effects on the adrenal gland are of particular interest. There is a sharp depres-

sion of adrenocortical function(8), accompanied by marked hypertrophy. The most likely explanation for this hypertrophy lies in release of pituitary from the inhibitory action of adrenal steroid with consequent over-production of adrenocorticotrophic hormone. Enlargement is greater than any usually obtained with stress. This may be explained, however, since the situation is somewhat unique in that the adrenal gland cannot limit continuing ACTH stimulation as it normally would by steroid production.

The mechanism of effect on adrenal function is not known. It is well established that cholesterol is at least a *potential* precursor for most adrenocortical steroids(20). In view of the demonstrated effectiveness of Δ^4-cholestenone in inhibiting cholesterol biosynthesis the present findings may indicate an *obligatory* role for cholesterol. Δ^4-cholestenone may actually be inducing a relative cholesterol 'deficiency'. The partial protective effect of exogenous cholesterol is compatible with this interpretation. On the other hand, since the biosynthetic pathways for cholesterol and for steroids are closely similar we may be dealing with a simultaneous interference with both and no conclusions regarding the role of cholesterol *per se* as an intermediate should be drawn. Finally, accumulation of dihydrocholesterol in the adrenal glands may be interfering with function mechanically or by adsorption on sites normally reserved for cholesterol.

The results obtained with Δ^4-cholestenone suggest that much valuable information about the role of cholesterol in body economy may be gained through use of this or similar inhibitors of cholesterol biosynthesis.

Summary. 1. Toxic effects of Δ^4-cholestenone fed at high dose levels to rats are described and data on absorption, distribution and fate of 4-C^{14}-Δ^4-cholestenone are presented. 2. A major effect of high dose levels of Δ^4-cholestenone is a marked inhibition of adrenal steroid secretion accompanied by marked hypertrophy of adrenal cortex. Some possible implications with respect to the physiological role of cholesterol in steroid biosynthesis are discussed. 3. Low dose levels are well tolerated, but lead to accumulation of dihydrocholesterol in serum in rats and, more strikingly, in man.

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Titrimetric Analysis of Calcium and Magnesium in Muscle.* (23881)

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Numerous methods of calcium and magnesium analysis for biological tissues, based on the metal chelating property of ethylenediamine tetraacetic acid (EDTA), have utilized the fact that above a pH of 12, EDTA chelates calcium more easily than magnesium. Therefore, in adding EDTA to a solution of magnesium and calcium, (plus any other cations equally well or more easily chelated by EDTA than calcium), a calcium ion indicator changes color at the precise point at which only magnesium remains unchelated, if the pH is greater than 12; subsequently, with the pH reduced to 10, a magnesium ion indicator changes color when further addition of EDTA has chelated the magnesium completely. Magnesium has been measured by EDTA titration using murexide as the calcium indicator and then completing the titration, on the same sample, with Eriochrome Black T as the magnesium indicator(1,2). However, murexide does not give a sharp visual end point. Recently, 2 new indicators, calcein(3) and 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid (Cal-red)(4), have been developed for calcium ion detection. Both give sharp end points in EDTA titrations. This paper deals with the use of these indicators, as well as Eriochrome Black T, in magnesium and calcium EDTA titration methods developed for muscle. In both methods, the cations were separated by precipitation from interfering substances in muscle ash solution. Calcium was precipitated with ammonium oxalate in acetic acid solution(5), subsequently the oxalate ion was destroyed by heating with perchloric acid(6). The calcium, in solution (pH above 12), was then titrated with EDTA, using either calcein or Cal-red as the calcium indicator. Magnesium was precipitated with 8-hydroxyquinoline (oxine) in ammonium hydroxide solution (7). The oxine was destroyed by heating with perchloric acid. EDTA was then added (pH above 12), until the precise end point for the calcium indicator (either calcein or Cal-red) was reached. The pH was then readjusted to 10, and the magnesium titrated with EDTA, using Eriochrome Black T as the magnesium indicator.

Materials. 1. *Oxine* 6% 8-hydroxyquinoline in 95% ethanol; 2. *Ammonium oxalate* saturated sol.; 3. *Methyl red indicator* saturated sol.; 4. *Calcein indicator* 2 mg% calcein (G. Frederick Smith Chemical Co., Columbus, O.) in 0.25 N KOH. Prepare weekly, keep refrigerated; 5. *Cal-red indicator* 40 mg % Cal-red sol. (Scientific Service Lab., Dallas, Texas). Prepare weekly, keep refrigerated; 6. *Eriochrome Black T indicator* 40 mg % Eriochrome Black T sol. (J. T. Baker Chemical Co., Phillipsburg, N. J.). Prepare weekly, keep refrigerated; 7. *Ammonium chloride-ammonium hydroxide buffer.* Add 35.5 g NH_4Cl to 224 ml conc. NH_4OH, dilute to 1 liter, keep refrigerated; 8. *Standard 2 mM calcium solution.* Dissolve 0.2000 g of $CaCO_3$ in min. volume of 1 N HCl, dilute to 1 liter; 9. *Standard 2mM EDTA solution.* Dissolve 0.75 gram (ethylenedinitrilo) tetraacetic acid disodium salt (Distillation Products Industries, Rochester, N. Y.) in water and dilute to 1 liter. Standardize against calcium sol. as follows: To 1 ml of standard 2 mM calcium sol., add 1 ml of 3 N KOH and 1 drop of calcein (or Cal-red) indicator; titrate with the EDTA solution until color changes from yellow-green to brown (If Cal-red is used, color changes from wine red to blue). (1 ml of 2 mM EDTA sol. is equivalent to 1 ml of 2 mM calcium sol.); 10. *Standard 2 mM magnesium solution.* Dissolve 50.83 g $MgCl_2 \cdot 6H_2O$ in water and dilute to 1 liter. The concentration of this stock sol. should be 250 mM. Dilute 8 ml of this stock sol. to 1 liter. Standardize against the EDTA sol. as follows: To 1 ml of the magnesium sol., add 1 ml ammonium chloride-ammonium hydroxide buffer and 1 drop Eriochrome Black T indicator; titrate with the standard 2 mM EDTA solu-

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tion until color changes from wine red to blue. (1 ml of 2 mM magnesium solution is equivalent to 1 ml of 2 mM EDTA solution.); 11. *Hydrochloric acid* 1N and 3N; 12. *Ammonium hydroxide* 1N and conc.; 13. *Potassium hydroxide* 3N; 14. *Acetic acid* 0.07 N; 15. *Perchloric acid* 70%; 16. Platinum crucibles (8 ml) for ashing procedure; 17. Conical centrifuge tubes (12 ml); 18. Metal shields (¾ by 4½ in.) for holding centrifuge tubes in sand bath; 19. Measuring pipettes, 1 ml, graduated to 0.01 ml, tip drawn out to deliver a 0.02 to 0.03 ml drop. The pipettes are coated with Desicote (Beckman Instruments, Fullerton, Calif.) to obtain a flat meniscus and facilitate splitting of drops. A thumbscrew pipette filler is used for filling and ejecting the titrating fluid; 20. Magnetic type stirring rods, about 1 cm long. Prepared by sealing No. 32 galvanized wire within capillary glass tubing (outside diameter 0.85 mm, Arthur H. Thomas, Philadelphia, Penn.). These rods, placed in the centrifuge tubes during the titrations, are agitated to give adequate mixing by moving a small magnet along the outside walls of the tubes; 21. Polyethylene reagent bottles.

Procedures. All volumes are approximate except those for standard solutions. *Calcium determination.* Ash muscle at 500°C (500-1000 mg wet wt) overnight in platinum crucible. Wash ash into centrifuge tube with 0.5 ml of 1 N HCl. Add 2 drops of methyl red indicator and then add dropwise 1 N NH_4OH until the color changes to yellow. Complete washing crucible into centrifuge tube with 3 ml of 0.07 N acetic acid. Add 1 ml of the ammonium oxalate solution, allow precipitate to form overnight. Centrifuge (3000 rpm, 10 minutes) and carefully remove supernatant. Dry precipitate by gentle warming and add one drop of 70% perchloric acid. Lower the tube slowly into metal shield embedded in a sand bath hot enough so that top part of tube is at least 200°C. After the white perchloric acid fumes cease to appear, remove tube from sand bath and dissolve residue in 2 drops of 3 N HCl. Add 1 ml of 3 N KOH and 1 drop of calcein (or Cal-red). Titrate with 2 mM EDTA to the appropriate change. In reading end points, the total volume should be 2 ml or more (add water if necessary). Compare with blank. Back titrations with the 2 mM calcium standard are sometimes helpful. *Magnesium determination.* Ash muscle (about 200 mg wet wt) as above. Wash into centrifuge tube with 0.5 ml of 1 N HCl and 1 ml of water. Add 0.5 ml of oxine and 0.5 ml of concentrated NH_4 OH, and allow precipitate to form overnight. Centrifuge and oxidize with perchloric acid as above. If color is not completely destroyed, repeat perchloric acid treatment. Dissolve residue in 2 drops of 3 N HCl. Add 1 ml of 3 N KOH and 1 drop of calcein (or Cal-red). Add 2 mM EDTA until the color just changes. Add water if necessary to make volume 2 ml. Compare with blank. Adjusting this end point is a critical step. It is sometimes made easier by using the 2 mM calcium solution. Readjust pH to 10 with addition of 1 ml of cold ammonium chloride-ammonium hydroxide buffer followed by 1 ml of 3 N HCl. Add 1 drop of Eriochrome Black T and titrate with 2 mM EDTA. Compare the end point with blank. For back titration, either the 2 mM calcium or magnesium standard may be used.

Results. Tables I and II show calcium and magnesium recoveries from solutions of known composition. With calcium:magnesium ratios of 1 to 10 and 10 to 1, there appeared to be no significant reciprocal interference in either determination. To test the possible interference of other muscle constituents, a pool of several muscles was ashed and dissolved in 1 N HCl. The calcium and magnesium concentrations of this muscle ash solution were determined. Known amounts of calcium and magnesium were also added to the muscle ash solution and were recovered as indicated in Tables I and II.

It is important in completely removing excess perchloric acid that the temperature be at least 200°C, since the constant boiling acid (71.6%) boils at 203°C. If some perchloric acid remains in the sample then insoluble potassium perchlorate will be formed upon KOH addition. However, even in the presence of potassium perchlorate, fairly sharp end points can be obtained.

Cyanide has been recommended to remove interfering cations for EDTA titrations using

Eriochrome Black T(1,2), calcein(3), and Cal-red(4). However, the addition of 1 ml of 0.5 N KCN to some muscle ash solutions just prior to the addition of the calcein (or Cal-red) indicator did not change the calcium and magnesium values.

Duplicate calcium and magnesium determinations were made on individual skeletal muscles from 13 frogs. The calcium was found to be 1.68 ± 0.07 (mean ± standard error of mean) millimoles/kg wet weight and the magnesium, 8.84 ± 0.31 (mean ± standard error of mean) millimoles/kg wet weight. These values yield a magnesium:calcium ratio of 5.26. The correlation coefficient was 0.79 with a probability value of 0.1%. This fairly high coefficient suggests some relationship between muscle calcium and magnesium. These findings are in reasonable agreement with previously reported values for frog muscle calcium(5,8) and magnesium(8).

In conclusion, these chemical methods offer the following advantages: the calcium does not have to be removed from the sample before determining magnesium; the procedures for calcium and magnesium are sufficiently alike so that both cation determinations can be performed concurrently with relative ease; and the actual titrations are relatively easy to perform, and do not require the use of a colorimeter.

TABLE I. Calcium Determinations.

No. of determinations	μmoles in sample Ca	μmoles in sample Mg	Avg μmoles Ca recovered	Stand. dev. of sample*
8	1	10	.96†	.05
8	10	1	9.88†	.14
3	0	2	.00	.00
10	1	1	1.02	.02
3	1	10	1.00	.02
5	2	0	1.99	.00
4	10	1	9.94	.07
4‡	1.3	9.59	1.32	.02
4‡	2.3	8.59	2.22	.10
4‡	3.3	7.59	3.30	.03

* Stand. dev. $= \sqrt{\frac{\Sigma (x - \bar{x})^2}{N}}$, where x is the individual value, $\bar{x}$ is the mean and N is the No. of values.

† These samples were titrated without precipitating the cation.

‡ These recovery experiments were carried out on 0.5 ml aliquots of a muscle ash solution to which known amounts of calcium and/or magnesium had been added. [Estimation of original calcium (1.30 μmoles) and magnesium (7.59 μmoles) of each 0.5 ml aliquot was calculated from the following observed concentrations: 2.60 ± 0.01 mM calcium (mean ± stand. error of mean) in 18 determinations, and 15.18 ± 0.09 mM magnesium (mean ± stand. error of mean) in 16 determinations.] The values given in the Table for calcium and magnesium content of these samples represent the sums of the amount originally estimated to be present plus the added amount, if any.

TABLE II. Magnesium Determinations.

No. of determinations	μmoles in sample Mg	μmoles in sample Ca	Avg μmoles Mg recovered	Stand. dev. of sample*
8	1	10	1.00†	.03
8	10	1	9.94†	.07
2	0	2	.00	.00
11	1	1	1.00	.01
2	1	10	.98	.02
6	2	0	1.98	.01
5	10	1	9.93	.10
4‡	7.59	3.3	7.60	.16
5‡	8.59	2.3	8.62	.24
5‡	9.59	1.3	9.66	.20

*, †, ‡ Same as Table I.

Summary. Relatively simple EDTA titration methods for the determination of muscle calcium and magnesium are given. Calcium is precipitated with ammonium oxalate; the oxalate is destroyed by heating with perchloric acid; and then the calcium is titrated with EDTA using either calcein or Cal-red as the indicator. Magnesium is precipitated with oxine; the oxine is destroyed by heating with perchloric acid; then sufficient EDTA is added in the presence of either calcein or Cal-red until the color of the indicator changes, after which the magnesium is titrated with additional EDTA using Eriochrome Black T as the indicator.

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Agglutination and Lysis of Erythrocytes by Lysozyme.* (23882)

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Utility of lysozyme as a bacteriolytic agent in the study of bacterial physiology and cell structure has been repeatedly demonstrated and recently reviewed(1). Though the specific substrate of lysozyme is unknown, this enzyme is classified as a mucopolysaccharidase. As such it should attack cells other than bacterial cells which contain superficially located mucoprotein. Erythrocytes are known to contain mucoprotein as blood group substances(2) and viral receptors(3). This article will report the hemolytic and hemagglutinating action of lysozyme upon these erythrocytes.

Materials and methods. Crystalline lysozyme was used throughout these studies and assayed 700,000 units/g(4). Dilutions of lysozyme were prepared in saline and assayed for their lytic activity by incubation for 2 hours at 37°C with an equal volume of a 0.25% suspension of the particular cells being studied. Hemagglutination assays were conducted in the same fashion but incubation was reduced to one hour. The red blood cell suspensions were pools of 3 or more donors except for sheep cells which were from individual donors or from a commercial source from an unknown number of donors. Pools were used to minimize difference between individuals, which, however, were later found to be minor. All titers represent the final concentration of lysozyme after addition of all reagents. Lytic titers do not represent complete lysis but are based upon the visual appearance of hemoglobin in the supernatant. Hemagglutination was determined by the cell settling pattern after refrigeration overnight and titers represent the greatest dilution of enzyme yet active. Kahn, Wassermann and 10 mm hemagglutination tubes were used without appreciable differences among the three.

Results. Lysis of erythrocytes by lysozyme was dependent upon the type of cell used (Table I). Human cells were consistently more sensitive than cells of other species yet apparently do not differ from one another. Pools of human AB blood were no different from other human types. Sheep and chicken cells were not as sensitive to lysis as human cells and rabbit cells were the most resistant of those studied. Lysis of chicken cells was frequently confirmed by microscopic examination since the hemoglobin is altered by lysozyme in such a way as to render it less visible. Assays of lysozyme with the Repaske technic(5) will increase the lytic titer against chicken cells approximately 2-fold. Similar experiments with other cell types have not been conducted.

TABLE I. Lysis of Erythrocytes by Lysozyme.

	Dilution of lysozyme							
Cell type	1:100	200	300	400	600	800	1200	1600
Human								
Group O	+	+	+	+	+	+	+	—
A	+	+	+	+	+	+	+	—
B	+	+	+	+	+	+	+	—
Sheep	+	+	+	+	+	±	—	—
Chicken	+	+	+	+	+	+	—	—
Rabbit	+	+	+	+	+	—	—	—

+ = lysis; — = no lysis; ± = lysis in some, but not all, repeats of the experiment.

When tubes of lysozyme solutions were placed in boiling water for a few minutes and then assayed the lytic ability of the enzyme for bacteria and erythrocytes was destroyed (Table II). During the heating process the solutions developed an hemagglutinating property. The destruction of the lytic ability and creation of the hemagglutinating activity were directly related to the duration of the heating and also to the decrease in the bacteriolytic titer. During the boiling process the solutions remain clear or become only slightly turbid.

Dilutions of lysozyme boiled for 20 minutes have the ability to agglutinate the cell types which were lysed by untreated lysozyme solutions (Table III). There was no direct re-

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TABLE II. Effect of Heating on Lysozyme Activity against Human O Erythrocytes.*

	Dilution of lysozyme in hundreds											Bacteriolytic units/g
	2	4	8	12	16	32	64	128	256	512	1024	
No treatment	L	L	L	L	L	—	—	—	—	—	—	700,000
Boiled 10 min.	L	L	L	H	H	H	H	H	—	—	—	20–40,000
" 20 "	L	H	H	H	H	H	H	H	H	H	H	5–10,000

* Individual, not pooled cells.
L = lysis; H = hemagglutination; — = no visible reaction.

TABLE III. Agglutination of Erythrocytes by Boiled Lysozyme.

Cell type	Dilution of lysozyme in thousands								
	13	26	51	102	205	410	819	1638	3277
Human Group O	+	+	+	+	+	+	—	—	—
" " A	+	+	+	+	+	+	—	—	—
" " B	+	+	+	+	+	+	—	—	—
Sheep	+	+	—	—	—	—	—	—	—
Chicken	+	+	+	+	—	—	—	—	—
Rabbit	+	+	+	+	+	+	+	+	—

+ = agglutination; — = no visible reaction.

lationship between lysis and agglutination since rabbit cells, the most resistant to lysis, were the most sensitive to agglutination. However, sheep cells were highly resistant to both of these properties of lysozyme solutions.

Discussion. Erythrocytes were seemingly more resistant to lysis by lysozyme than most Gram positive bacteria, requiring concentrations of 0.05 to 0.25 mg/ml as compared to μg/ml quantities for bacterial suspensions. It is doubtful that the 2 systems may be compared so directly, however; for the nature, amount and availability of the enzyme substrate in these systems is still unknown.

It is not yet clear if lysozyme is actually acting upon mucoprotein of the erythrocytes since, in data not presented, sublytic exposure of chicken cells to lysozyme with subsequent removal of the enzyme by repeating washing of the cells did not produce cells with diminished influenza virus A(PR-8) hemagglutinating titers. Likewise, human cells similarly exposed to lysozyme were agglutinated to the same titer as normal cells by specific immune serum.

Hemagglutination by lysozyme has not been described previously although agglutination of bacteria has been observed(6,7,8). These latter were largely random studies not given detailed study. It is possible that agglutination and hemagglutination occur by the same or similar mechanisms since weakly bacteriolytic preparations of lysozyme have been more reactive in bacterial agglutination. However, in one instance the ability of lysozyme to flocculate bacteria was destroyed by boiling(9).

The remarkable dilutions of denatured lysozyme which agglutinated red blood cells were unparalleled even by phytoagglutinins which are usually more active than immune sera.

Summary. Two new observations have been presented; lysis and agglutination of erythrocytes by lysozyme. The hemagglutinating reagent may be prepared by boiling the hemolytic enzyme solution.

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Some Metabolic Studies of γ-Aminobutyric Acid.* (23883)

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Relatively large quantities of γ-aminobutyric acid (GABA) have been detected by us in an easily extractable form in the central nervous system of representative members of fish, reptilian, avian, marsupial, and mammalian species, but not in other tissues. GABA was the only extractable ninhydrin-reactive material which showed progressive increases at successive developmental stages in extracts of brains of chick embryo, mouse, salamander, and frog. Both GABA and L-glutamic decarboxylase, the enzyme which catalyzes formation of GABA from L-glutamic acid, were present in uniquely high concentrations in gray matter of the central nervous system. GABA transaminase, an enzyme found in brain as well as other tissues, catalyzes transamination of GABA with α-ketoglutarate (αKG). There exists the possibility that in brain a shunt operates around the αKG oxidase system of tricarboxylic acid cycle by which αKG could be withdrawn through formation of glutamic acid by transamination with GABA, and the carbon chain of GABA could enter the cycle at the succinate level (see reference 1 for pertinent literature). The results of present experiments indicate that the above formulation, based on enzymatic studies, is actually consistent with reactions which occur *in vivo*.

Methods. Fresh specimens were fixed in 75% alcohol immediately after dissection and rapid weighing on torsion balance. Extracts were prepared by homogenizing the tissue in alcohol with ground-glass, motor driven homogenizer, clarifying by centrifugation, and evaporating supernatant fluid in 20 ml beakers under infra-red lamp in air stream of a fan. Each residue was taken up in saturated aqueous solution of picric acid (100 μl/100 mg of original fresh weight of tissue) and the suspension centrifuged. Usually a total of 75 μl of supernatant was placed in 3 applications of 25 μl each onto 18″ x 22″ sheets of Whatman No. 1 filter paper, although occasionally 20 μl of supernatant was placed on 9″ x 11″ sheets. Descending 2-dimensional chromatography was performed in water-saturated phenol followed by water-saturated lutidine. The picric acid migrated with solvent front in lutidine and did not interfere with subsequent detection of ninhydrin-reactive constituents, visualized by spraying with 0.1% ninhydrin in butanol. In some instances quantitative estimations of amounts of amino acids were made by eluting the spots with water from chromatograms and immediately reading the colors in Beckman DU spectrophotometer at 570 mμ. Standard curves were prepared from suitable mixtures of amino acids chromatographed in the same manner and at same time. Radioautographs of some of chromatograms were prepared by placing unsprayed sheets in contact with Type K Industrial X-ray film for suitable time. Quantitative measurements of radioactivity of eluates of areas corresponding to some amino acids were made by standard technics in a gas-flow counter. Other procedures will be described in sections dealing specifically with their use.

Results. Conversion of carbon chain of L-glutamic acid to GABA in brain of intact mouse; inhibition by thiosemicarbazide. Previous work has shown that the carbon chain of uniformly C^{14}-labeled glutamic acid can be converted to carbon chain of GABA *in vitro* by mouse brain preparation and studies were made of properties of L-glutamic acid decarboxylase, the enzyme which forms GABA from glutamic acid(1). The following experiments show that glutamic acid is, indeed, a major precursor of GABA in the intact brain of living animal.

Approximately 35 γ of L-glutamic acid-U-C^{14} (11.3 mc/mM; Nuclear Inst. and Chem. Corp.) contained in 0.03 ml of water was in-

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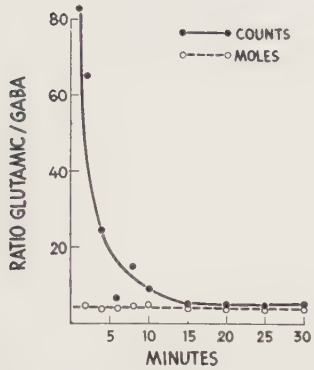


FIG. 1. Glutamic acid/GABA ratios in extracts of brains of mice at various times after intracer. inj. of L-glutamic acid-U-C^{14}. The 30 min. value is the avg for 5 animals, all other points are for individual animals.

jected intracerebrally into each of 9 mice of Webster-Swiss strain weighing 15 to 17.5 g. The mice were sacrificed by cervical dislocation at 1, 2, 4, 6, 8, 10, 15, 20, and 25 minutes after injection and the brains analyzed in usual manner. The results were expressed in ratios of radioactivity found in glutamic acid to that observed in GABA. The ratios of radioactivities decreased from initial value higher than 80 to that of approximately 4 within 15 minutes after injection (Fig. 1). The molar ratios of glutamic acid to GABA also were approximately 4. Therefore, specific activities (counts/min/μM) of GABA and glutamic acid were essentially the same after 15 minutes. These results show that the carbon chain of GABA can be formed from the carbon chain of L-glutamic acid in brain *in vivo*.

Chromatograms and radioautographs of extracts of the 4 and 25 minute samples are shown in Fig. 2 and 3, 4 and 5, respectively. All compounds detected at later times on these radioautographs were radioactive even in the one minute sample, indicating an extremely rapid entry of glutamic acid carbon into metabolic pathways. A very high level of radioactivity was found in glutamine at all times. Increase in radioactivity of GABA with time is seen by inspection of photographs of the radioautographs. An experiment was then performed in which 5 mice received 0.4 mg of thiosemicarbazide intraperitoneally 15 minutes prior to intracerebral administration of 0.03 ml of C^{14}-labeled glutamic acid as described previously. The animals were killed and the brains removed for analysis 30 minutes after glutamic acid was given. No convulsions occurred during the study. A group of control mice received only glutamic acid 30 minutes prior to removal of brains. The results in Table I show that the amount of glutamic acid relative to GABA was not increased significantly by thiosemicarbazide. However, a highly significant increase in ratios of radioactivities of these 2 amino acids was noted, showing decreased rate of flow of isotope from glutamic acid into GABA. These results confirmed, by measurements in intact animal, the results obtained by Killam(2,3) who showed that convulsant hydrazides inhibited glutamic acid decarboxylase activity in brain, and inferred that the convulsant ac-

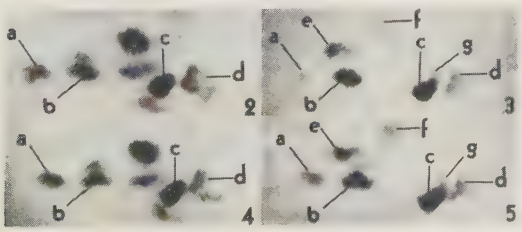


FIG. 2-5. Chromatographs and radioautographs of extracts of mouse brain after intracer. inj. of L-glutamic-U-C^{14}. Fig. 2 and 4 are the chromatograms prepared at 4 and 25 min., respectively, and Fig. 3 and 5 are the corresponding radioautographs. a, GABA; b, glutamine; c, glutamic acid; d, aspartic acid; e, probably pyrrolidonecarboxylic acid (formed from glutamine); f, unknown; g, succinic acid.

TABLE I. Glutamic Acid/GABA Ratios in Extracts of Mouse Brains 30 Minutes after Intracerebral Injection of L-glutamic Acid-U-C^{14}.

Treatment	Glutamic acid/GABA	
	Molar	Radioactivity
Controls	3.9	4.7
	4.6	4.0
	3.0	3.4
		4.2
	2.5	4.0
Avg	3.5	4.1
Thiosemicarbazide*	4.0	7.1
	3.4	15.9
	3.5	7.6
	4.2	11.2
	3.7	13.1
Avg	3.8	11.0

* 0.4 mg/mouse given intraper. 15 min. prior to intracer. inj. of glutamic acid.

TABLE II. Radioactivity of Succinic and α-Ketoglutaric Acids in Urine of Rats after Administration of γ-Aminobutyric Acid-1-C^{14}.

Exp. No.	Radioactive compound administered	Dose*	Amt of catabolite administered (mg)	Catabolite isolated	Specific activity of isolated catabolite†
1	Acetate-1-C^{14}	1.3×10^7	400	α-Ketoglutarate‡	180
			"	Succinate§	46
2	γ-Aminobutyric acid-1-C^{14}	9.8×10^6	"	α-Ketoglutarate	53
			"	Succinate	240

* Disintegrations/min. † Disintegrations/min./mMole $\times 10^{-3}$. ‡ Isolated as the 2,4-dinitrophenylhydrazone. § Isolated as succinic acid.

tion of these drugs was related to this effect.

Conversion of the carbon chain of GABA to succinic acid in the intact rat. One and two-tenths mg of GABA-1-C^{14} (Tracerlab) and 400 mg of succinate (neutralized with NaOH) in 5 ml of H_2O were injected intraperitoneally into a female Sprague-Dawley rat (250 g) fasted previously for 24 hours. The urine was collected 18 hours, made alkaline, and extracted continuously for 10 hours with ether. The aqueous solution was acidified and then extracted for another 12 hours with fresh ether. The extract was dried over sodium sulfate, filtered, and evaporated to dryness. The residue was recrystallized from ether-benzene mixture, dissolved in water, treated with Norit, filtered, evaporated, and dried. In this manner 73 mg of white crystals were obtained and sublimed at 100° at 1 mm pressure to obtain 69 mg of white solid succinic acid. To this was then added 69 mg of inert succinic acid and the mixture recrystallized 3 times from ether-benzene mixture to constant specific activity. In a similar experiment the same amount of GABA-1-C^{14} was injected simultaneously with 400 mg of αKG into another rat. Urines were collected, treated with excess of 2-4 • dinitrophenylhydrazine in 2N HCl, the precipitate collected and recrystallized repeatedly from 10% ethanol and ethyl acetate-chloroform mixture to constant specific activity. Similar experiments were performed in other rats using acetate-1-C^{14} and cold succinate or αKG.

The results are shown in Table II. Injection of labeled acetate together with either succinate or αKG gave a 4-fold higher specific activity in αKG than in the succinate isolated. This result is in keeping with the known facts of tricarboxylic acid cycle, the carbon from acetate reaching succinate only after passing through αKG. On the other hand, when GABA-1-C^{14} was administered with succinate or αKG the specific activity of the isolated succinate was approximately 4.5 times greater than that of the αKG. The latter result indicates that the carbon of GABA probably enters the tricarboxylic acid cycle at the succinate level. That the conversion to succinate represents a pathway of metabolism of GABA in the intact animal is indicated by the fact that as much as 2% of the radioactivity of GABA administered was recovered in the succinate isolated. The latter results are consistent with the findings on metabolism of the closely related substances, β-alanine (4) and δ-aminovaleric acid (5), and suggest that transamination and subsequent oxidation of the carbon chain are major metabolic reactions of GABA *in vivo*. The isotope-trapping technic described above has been used previously for study of lysine metabolism (6).

Metabolism of γ-aminobutyric acid-1-C^{14} after intracerebral injection in mice. Seven male Swiss mice weighing approximately 25 g were injected intracerebrally with 0.03 ml aqueous solution containing approximately 560,000 counts of GABA-1-C^{14} (250 γ). The mice were killed by cervical dislocation at different intervals after injection, the brains removed immediately, and prepared for analysis. Total activity recovered in alcoholic extracts is shown in Table III. The highest recovery of radioactivity, 44% of that administered, was achieved in the mouse killed at 11.5 minutes after injection. The proportion of radioactivity recovered decreased at

TABLE III. Radioactivity in Extracts of Mouse Brain and in GABA at Various Times after Intracerebral Administration of GABA-1-C^{14}.*

Min. after inj.	Total counts in extract (counts/min.)	Counts in GABA (counts/min.)	% of total counts of extract in GABA
	× 1000	× 1000	
2	188.8	183.0	97
5	146.4	117.0	80
11.5	244.0	211.0	87
27	80.4	33.0	41
61	18.4	6.0	32
120	10.8		
240	3.5		

* Each mouse received an intracer. inj. of 0.03 ml of solution containing 560,000 counts/min. of GABA-1-C^{14}. Radioactivity was measured in a gas-flow counter.

rapid rate, only 3% of injected amount being detected after 1 hour. After preparation of 2-dimensional chromatograms and development of radioautographs of these chromatograms, the area containing GABA was cut out, eluted, and a suitable aliquot counted. The percentage of total activity found in GABA decreased after 11.5 minutes. Even 2 minutes after injection, radioactivity was detectable in aspartic and succinic acid areas and in glutamine. At 27 minutes the glutamine showed greatest radioactivity of constituents other than GABA. Radioactivity of all observable constituents decreased in brains obtained one and 2 hours after injection, glutamine and GABA remaining the most highly radioactive materials in the extracts.

Metabolism of γ-aminobutyric acid-1-C^{14} during incubation with brain minces. Confirmation of above results was obtained in experiments with brain minces. Two whole mouse brains were minced into 3 ml of glucose-containing bicarbonate-saline(7) in each of four 20 ml beakers. To each beaker was added 0.02 ml of GABA-1-C^{14} (390,000 counts), and incubated at 30° in 95% oxygen and 5% carbon dioxide. At 15, 30, 60, and 90 minutes, respectively, the contents of individual flasks were poured into 90 ml of boiling 95% ethyl alcohol contained in ground glass homogenizer and the samples homogenized and worked up for analysis in usual manner. After chromatography and radioautography the spots corresponding to aspartic, glutamic, and succinic acids and GABA and glutamine were eluted and counted. The results are shown in Table IV. The major radioactive areas on the radioautographs were attributable to GABA, aspartate, glutamate and glutamine at all time intervals. With the exception of GABA, the largest amount of radioactivity was found in the aspartate. Finding of the label in succinate and amino acids which can arise from the keto-acids of tricarboxylic acid cycle is consistent with experiments on the intact animal. Since completion of above experiments, similar findings have been made with guinea pig brain slices and rat brain mitochondria(8).

Correlation of biochemical and physiological findings. Although the chief pathways of formation of GABA in nervous tissue and its utilization were known(1), there were no clues from the biochemical information regarding the possible function and importance in the central nervous system of the uniquely high concentrations of GABA and of the enzyme which forms it from glutamic acid. An extensive series of studies by Florey (see 9 for references) on a factor isolated from beef brain which has potent physiological activity

TABLE IV. Distribution of Radioactivity at Various Times after Incubation of Mouse Brain Minces with GABA-1-C^{14}.

Min. of incubation	Total counts in alcoholic extract (counts/min.)	Total counts in constituents of picric acid filtrates* (counts/min.)				
		GABA	Aspartic acid	Glutamic acid	Glutamine	Succinic acid
15	389,600	374,000	5,770	1,425	483	544
30	376,600	311,000	7,840	1,140	149	1,605
60	289,600	229,500	7,550	2,595	4,170	1,180
90	243,800	197,500	10,470	1,960	6,080	763

* The substances were visualized by radioautography of 2 dimensional chromatograms of the picric acid filtrates, eluted, and suitable aliquots were counted. Amount of radioactivity in the picric acid precipitate was not determined.

in a number of test systems has culminated recently in the identification of this factor as GABA(10), and a number of lines of evidence have been adduced in favor of assigning to it the role of a transmitter substance of inhibitory neurons in decapod crustaceans (9). GABA appears to act by increasing the permeability of the synaptic membrane of the crayfish stretch receptor to chloride and potassium, but not to sodium, a mode of action also ascribable to the inhibitory axon which forms synapses with the dendrites of the receptor(11,12). It is not known whether the pharmacological effects of GABA are attributable to the interaction of GABA itself with receptor sites or whether some metabolite which is formed rapidly from GABA is involved. It was reported recently that γ-guanidobutyric acid is found in mammalian brain(13) and that it can be synthesized in kidney from GABA and an amidine donor (14), but GABA has proven to be much more active in the crustacean test system than the guanido derivative(11,12). Although γ-butyrobetaine could conceivably be formed from GABA, neither betaine(15) nor γ-butyrobetaine (Florey, personal communication) affected the stretch receptor preparation, nor was it possible to show the presence of γ-butyrobetaine or its esters in brain extracts (unpublished, this laboratory) by a combination of isolation and paper chromatographic procedures(16). Succinic semialdehyde, formed on transamination of GABA with αKG(17), is oxidized readily by brain preparations(8), but its effectiveness in pharmacological test systems has not been reported.

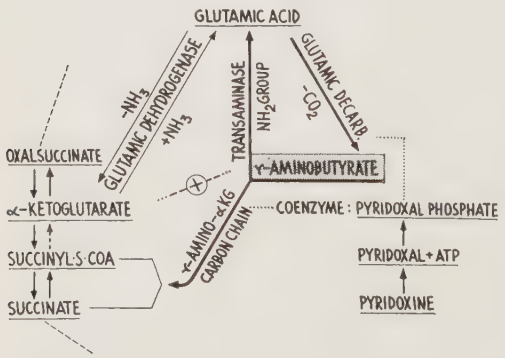


FIG. 6. Known metabolic relationships of GABA.

Since GABA may play an important role in regulating activity in the central nervous system, it is important to consider the various factors which might be involved in maintenance of given, relatively constant amounts of this substance. Widely differing levels of GABA have been found in various areas of the brain. The coordination of the metabolic sequences shown in Fig. 6 requires the proper functioning of several enzyme systems which could be affected by genetic or environmental factors.

Both glutamic acid decarboxylase(1) and GABA transaminase(18) require for activity pyridoxal phosphate, which can be synthesized in brain from ATP and pyridoxal(1). Work to date indicates that the apoenzyme of glutamic decarboxylase has a lower affinity for the coenzyme than that of GABA transaminase(18). Observations in many laboratories have established a relationship of convulsive seizures to pyridoxine deficiency in experimental animals and in human beings. Dietary pyridoxine deficiency of insufficient severity to produce seizures in rats resulted in a marked but readily reversible decrease in the activity of glutamic decarboxylase(1). Convulsant hydrazides, compounds which react with the coenzyme, have been shown to produce decreases in the activity of glutamic decarboxylase and the content of GABA in the brains of rats(2) and in the present study to decrease the rate of formation of GABA from glutamic acid *in vivo* in mice. A good correlation was obtained between electrically detected seizure activity and decreases in glutamic decarboxylase activity in affected areas in brains of cats(3). Topical application of GABA to the cortex has reversed seizures in cats induced by parenteral administration of hydrazides,‡ while the placing of solutions of GABA on the cortex of normal animals has been shown to have a depressant action(19-21). Convulsions in mice produced by the injection of methoxypyridoxine could be prevented completely by the injection of small amounts of pyridoxine or large amounts

‡ Dasgupta, S. R., Killam, E. K., Killam, K. F., presented before the Am. Soc. for Pharm. and Exp. Therap., Baltimore, Sept. 6, 1957.

of GABA (Gumnit and Gammon, personal communication). All of the above results fit the tentative hypothesis that seizure activity in pyridoxine deficiency and hydrazide poisoning results from the decrease of activity of the glutamic acid decarboxylase with a consequent decrease in ability to produce GABA. Studies have not yet been made of the relative degree of inhibition of glutamic decarboxylase and GABA transaminase by pyridoxine deficiency or by the convulsant hydrazides. However, the presently available results would suggest that glutamic decarboxylase is preferentially inhibited whenever pyridoxal phosphate becomes a limiting factor.

Picrotoxin was found to antagonize the inhibitory action of GABA on both the crayfish stretch receptor and heart(9). It is interesting that smaller amounts of both semicarbazide(22) and picrotoxin(23) were required to produce seizures in monkeys with experimentally induced epilepsy than in controls. GABA and acetylcholine have been found to be antagonistic to each other in a number of test systems(9). It appears possible that the glutamic decarboxylase-GABA-GABA transaminase system might be a companion system to the choline acetylase-acetylcholine-cholinesterase system in regulation of activity in the central nervous system (see 24 for discussion of role of acetylcholine in seizures). The balance between these systems, rather than the absolute amount of any single substance or enzymatic activity, might be important in the regulation of activity in a given region of the central nervous system and disturbances in this balance might be involved in at least some cases of clinical epilepsy. It is possible to begin the analysis of the action of a number of pharmacologically active materials in terms of imbalances produced by their action on specific enzymes in the GABA and acetylcholine sequences.

The pH activity relationships of cerebral glutamic decarboxylase(1) and GABA transaminase§ are shown in Fig. 7. The results for each of the enzymes are plotted as % of maximal activity under optimal conditions of assay. Glutamic decarboxylase possesses maximal activity at pH 6.5, GABA transaminase at 8.2. The two curves intersect (possess same percentage of maximal potential activity) at pH 7.5, which might be assumed to be close to intracellular pH of brain. It is apparent that small shifts in pH in the physiological range could result in large changes in relative activity of the 2 enzymes and so might alter the content of GABA at the loci where its physiological effects are exerted. *Intracellular* acidosis would tend to increase the content of GABA and alkalosis to decrease it. *Metabolic* acidosis has been generally found to decrease the incidence of epileptic seizures and some types of experimental seizures and alkalosis to increase them (see 25 for pertinent references).

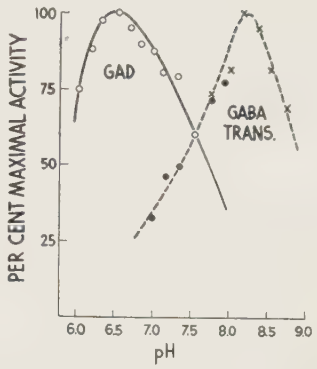


FIG. 7. pH activity relationships of cerebral glutamic decarboxylase and GABA transaminase. ○——○ and ●– – –●, phosphate buffer; ×– –×, borate buffer.

Summary. In agreement with previously reported enzymatic studies, experiments with intracerebrally administered L-glutamic-U-C^{14} in intact mice showed that carbon chain of L-glutamic acid is a source of the carbon chain of GABA formed in brain and that thiosemicarbazide decreases the rate of conversion of cerebral glutamic acid to GABA. It was shown that carbon of GABA can enter the tricarboxylic acid cycle at the succinate level in intact rats in experiments with intraperitoneally administered GABA-1-C^{14} employing the isotope trapping technic. Experiments in which GABA-1-C^{14} was injected intracerebrally to mice, or incubated with minces of mouse brain, indicated that utiliza-

§ The details of properties of GABA transaminase are being reported elsewhere.

tion of GABA in brain probably takes place by the same mechanism as in the whole animal. The results of present and past biochemical studies can be correlated with relevant physiological data.

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Hemadsorption (Adsorption-Hemagglutination) Test for Viral Agents in Tissue Culture with Special Reference to Influenza. (23884)

ALEXIS SHELOKOV,† JOHN E. VOGEL† AND LOTTA CHI (Introduced by D. J. Davis)
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In a recent publication(1) we described "adsorption-hemagglutination" which occurs when appropriate erythrocytes are added directly to monkey kidney cell cultures infected with influenza A virus. The present report indicates some practical applications of this reaction, now designated as *hemadsorption,* in the diagnosis of influenza, mumps, and CA (croup-associated) virus infections. In addition, its usefulness with other viruses was investigated using prototype strains in tissue culture systems.

Materials. Several types of trypsinized monolayer cell cultures propagated in usual growth media were used. Most experiments employed monkey kidney (MK) cells prepared by modification of standard methods (2); chick embryo and monkey testicular cells were similarly processed; amnion cultures were made according to procedure developed in this laboratory(3); HeLa and bovine kidney cells were purchased from Microbiological Associates. Tissue culture (TC) tubes

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TABLE I. History of Virus Strains at Time of Hemadsorption Test.

Virus	Designation	Material tested	Source	No. of strains tested
Influenza	A/US/57	Throat swab	Unit*	33†
Mumps	26727 (urine)	MKTC	J. P. Utz	1
	FS-2064	Throat swab	Unit	1
CA (croup-associated)	Greer	MKTC	R. M. Chanock	1
	FS-CA	Throat swab	Unit	5
Sendai	Prototype	MKTC	R. M. Chanock	1
	Sendai-CDC	Egg	K. K. Takemoto	1
NDV (Newcastle)	F1234-13	Egg	L. Loomis	1
Vaccinia	MK 22	MKTC	J. Warren	1
Simian, SV 2,4,5,12,15	Prototypes	MKTC	C. P. Li	1 each
EMC	Entebbe	Mouse brain	L. Kilham	1
Herpes simplex	Zacchardi	HeLa TC	P. Halonen	1
Poliovirus 1,2,3	Prototypes	MKTC	Unit	1 each
Coxsackie A9, B1-B5	"	MKTC	Unit	"
ECHO 1-11	"	MKTC	K. Habel	"
Adenovirus 1,2,4,6,7,8	"	HeLa TC	W. P. Rowe	"

* *Unit* refers to the Laboratory Unit, Epidemiology Section, Lab. of Infect. Dis.
† 7 strains related to A/Md/1/55; 26 strains related to A/Jap/305/57.

were maintained in suitable media enriched with serum, but at time of inoculation were changed to Eagle's basal medium (4) with 2% fresh glutamine usually without serum. Histories of virus strains tested for hemadsorption are recorded in Table I. Influenza immune rooster serums were produced in our laboratory or obtained as standard merthiolated typing serums from International Influenza Center for Americas of PHS Communicable Disease Center. Dr. R. M. Chanock and Dr. J. P. Utz kindly supplied us with guinea pig and monkey antiserums used in CA virus and mumps identification. Citrated guinea pig, chicken, rhesus monkey, human O, and defibrinated sheep erythrocytes were washed 3 times and made to 0.4% suspension by volume in sterile physiological saline or balanced salt solution (BSS). Antibiotics were added to some batches.

Methods. Virus isolation procedure by hemadsorption test consisted of inoculating TC tubes with 0.1-0.2 ml of specimen material after replacing fluid with Eagle's basal medium. Tubes were incubated at 37°C and, at least with influenza field isolates, early demonstration of hemadsorption was enhanced by rotating them in a drum. After appropriate period of incubation 0.2 ml of erythrocyte suspension was added to each tube whether or not cytopathic effect (CPE) was observed. The tubes were left in inclined position 3 to 5 minutes, revolved to resuspend settled red blood cells, and examined under low magnification (×100-150). The hemadsorption test was read *negative* when unclumped erythrocytes were observed floating in supernatant without significant adsorption to TC cell sheet. Negative tubes were replaced in revolving drum and periodically examined 6-8 days. Because of inevitable lysis of erythrocytes on incubation they were replenished every 2 days. Test was read *positive* when there was evident adsorption of variable number of erythrocytes onto monolayer sheet in characteristic pattern described below. The tissue cell sheet was then scraped off and cellular debris, including erythrocytes, were homogenized in tissue grinder or mortar, using supernatant as diluent. One-tenth ml of homogenate was added to each of several monkey kidney tubes for passage. Because on second passage the CPE was invariably observed, typing was done after early cytopathic changes appeared. With mumps and CA viruses, the supernatant alone was used for passage and identification. We used one or both of following procedures to type the isolates. 1) *Hemadsorption-inhibition test.* After removing fluid from inoculated and control tubes they were washed once with BSS. Sixth-tenths ml of BSS were added

TABLE II. Hemadsorption Reaction with Influenza and Mumps Viruses.*

TC system	Monkey kidney					Human amnion		Monkey testis	Bovine kidney
Erythrocytes	Guinea pig	Chick	Sheep	Human O	Rhesus	Guinea pig	Chick	Guinea pig	Guinea pig
Influenza A	+	+	+	+	+†	0	0	0	0
Mumps	+	+	+	+	+	+	+	+	+

* + = Hemadsorption demonstrated. 0 = Hemadsorption not demonstrated, but no evidence of viral propagation.

† Not all field isolates positive.

to each tube to be serum treated and 0.8 ml BSS to uninoculated, virus control, and isolate control tubes. At least 2 tubes in each set were treated with appropriate antiserums in 0.2 ml amounts of 1:10 dilution. After 15 minutes incubation in slanted rack at room temperature each tube was treated with erythrocyte suspension, returned to the rack for about 3 minutes, and then examined under microscope. 2) *Neutralization-hemadsorption test.* Material from initial passage and serum were diluted 1:10 and 0.1 ml amounts of each was inoculated into several cell cultures. The tubes were incubated either until early CPE appeared in control tubes or for time estimated from first passage to allow viral propagation. Erythrocyte suspension was then added and tubes examined. Comparative studies of influenza isolates in embryonated eggs, employed technics in standard use at NIH (Eddy, B., unpublished). After 2 passages of each specimen in 10-11-day-old embryos, the fluids were tested for hemagglutination by the Salk pattern test(5). Positive specimens were titrated and typed by hemagglutination-inhibition using rooster immune serums treated with *V. cholerae* filtrate.

Results. Influenza. We demonstrated hemadsorption in MK cultures infected with influenza A virus using erythrocytes of guinea pig, chicken, sheep, human type O, and rhesus monkey origin (Table II). Attempts in other cell cultures including human amnion, monkey testis and bovine kidney have been unsuccessful apparently due to failure of influenza viruses to propagate in these TC systems. Tubes inoculated either with TC adapted strains or specimens from suspected cases were first examined for hemadsorption after 40 hours of incubation. Fig. 1a and 1b show a positive early reaction in different portions of the tube: the cell sheet is studded with adherent guinea pig erythrocytes in an easily recognizable characteristic pattern of *clusters,* oval or elongated *rosettes* (formed when individual or adjacent cells are surrounded by erythrocytes), and *chain formations* (Fig. 1a), some of which apparently follow lines of cleavage between MK cell groupings in *streamer* effect (Fig. 1b). These formations are commonly seen in tubes with limited viral propagation, but in later stages the entire sheet may be literally plastered with adsorbed red blood cells (Fig. 1c). At this time it is not uncommon to find visible *clumps, i.e.* irregular aggregates of truly hemagglutinated erythrocytes, in the fluid phase indicative of viral release from TC cells. For comparison, a similarly treated uninoculated MK cell culture of same age and a poliovirus-infected tube without hemadsorption are shown in Fig. 2a and 2b. The erythrocytes seen in these 2 photographs were actually slowly drifting across the field. CPE due to influenza field strains often did not appear on initial passage, although presence of hemadsorption in infected cultures indicated viral propagation in tissue cells. Because at this stage the nutrient fluid generally did not contain demonstrable virus, infected TC cells had to be included in the passage material. When specific CPE was observed on initial examination in a large enough number of tubes, the hemadsorption-inhibition test was done upon demonstration of hemadsorption in some cultures, resulting in positive identification of field strains in less than 2 days. Alkaline pH of TC medium exerts a marked effect on cultivation of influenza virus. Table III summarizes results of experiment in which the same known positive throat washing specimen was inoculated into 3 groups of MK tubes ad-

justed to pH 6.5, 7, and 8. Intermediate pH delayed viral propagation, while higher pH actually prevented demonstration of the virus in the field specimen. In addition, a MK-established influenza virus strain, previously isolated from this field specimen, was titrated at pH 6.5 and 8 and 100 times more virus was demonstrated at lower pH. This sug-

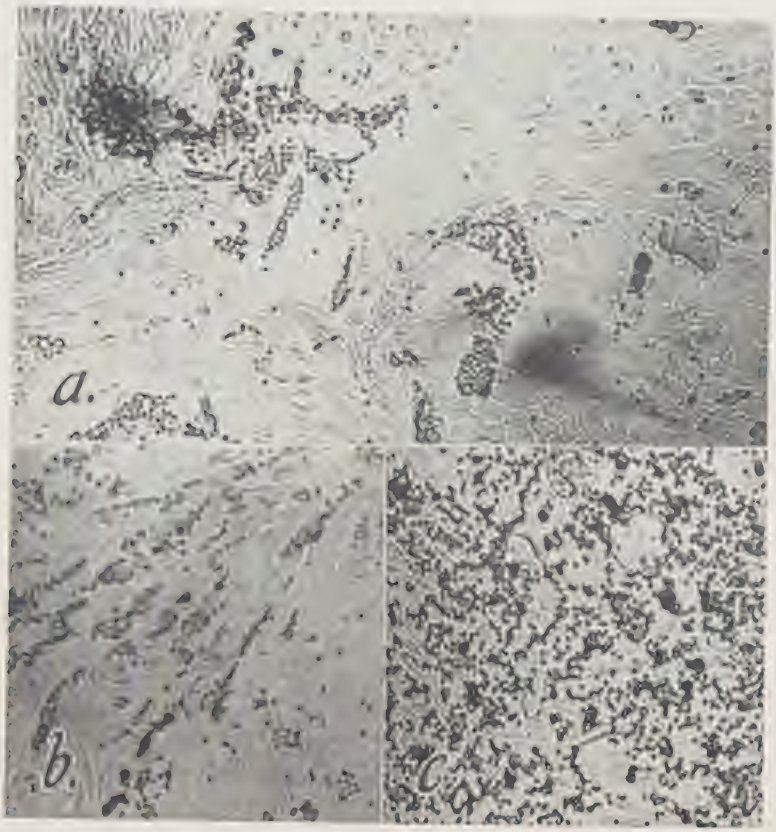


FIG. 1. Influenza A virus in MK culture. Hemadsorption with guinea pig erythrocytes at 40 and 100 hr (×105). a. Early effect (before CPE): clusters, chains, rosettes. b. Same tube, another field: streamers. c. Late effect (after CPE).

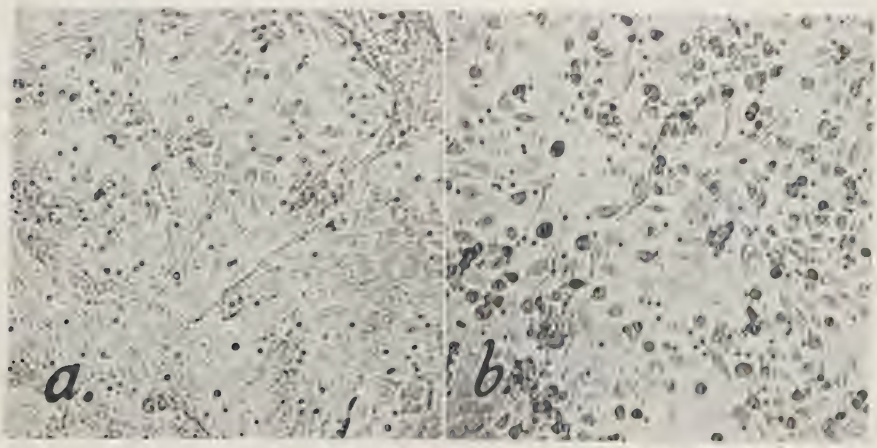


FIG. 2. MK cultures *without* hemadsorption of guinea pig erythrocytes (×105). a. Uninoculated control. b. Poliovirus, type 1 (early CPE).

TABLE III. Effect of pH of MK Maintenance Medium on Detection of Influenza Virus in Throat Washing Specimen by Hemadsorption Test.

	Days after inoculation		
	2	4	7
pH 6.5	+	+	+
7.0	0	+	+
8.0	0	0	0

+ = Hemadsorption demonstrated.
0 = " not demonstrated.

TABLE IV. Isolation of 1957 Influenza A Strains by Hemadsorption and Egg Technics.

Specimen group	No. of specimens	Hemadsorption positive	Embryonated egg positive	Total positive specimens
I	23	8	8	8
II	54	11	2	12*
III	16	2	0	2
Total	93	21	10	22*

* Includes 1 specimen negative in TC but positive in eggs.

gests that as much as 2 logs of infectious virus contained in aliquot of field specimen can be suppressed in TC by unfavorable pH.

An important demonstration of practical usefulness of hemadsorption test for influenza virus comes from its efficacy as compared to embryonated egg procedures. Table IV summarizes results of inoculation of both isolation systems with specimens from 3 outbreaks of Asian influenza in the United States. The first group of 23 was collected and stored under optimal conditions of CO_2 ice and 8 viral strains isolated from the same 8 specimens by both technics. Using hemadsorption test 6 of 8 strains were detected at 40 hours (the other 2 were demonstrated at 24 and 72 hours). The second and third groups of specimens, although properly collected, were subjected to thawing and freezing and storage at –20°C, undoubtedly with some loss of the infective virus. From these groups 13 strains were isolated and typed by hemadsorption reaction and only 2 by egg technic. In group II there was a single egg positive specimen missed in TC, but 10 other TC isolates were not detected in eggs. Thus, of 93 specimens tested at same time by both methods, 21 were found positive by TC-hemadsorption and only 10 by egg-HA systems.

We have never failed to type a strain of influenza virus by this technic. The reaction is apparently specific and heterologous immune serums produced in roosters, monkey, rabbits, and guinea pigs against other types of influenza, Coxsackie A and B, mumps, ECHO, CA, and polioviruses did not inhibit it.

Mumps. Studies with mumps virus were restricted to 2 strains. A recent isolate from patient's urine by Utz and coworkers(6) on propagation in MK cells produced strongly positive hemadsorption. The other strain was isolated and typed by hemadsorption reaction from routine throat swab of clinical case. This virus was reisolated 3 times in MK and amnion cultures and repeatedly identified as mumps by neutralization-hemadsorption and hemadsorption-inhibition tests. Fig. 3b shows early characteristic hemadsorption pattern when no CPE due to mumps virus could be detected, (Fig. 3a) and Fig. 3d shows effect 24 hours later when probable CPE was noted (Fig. 3c). Unlike influenza virus, mumps readily propagated in human amnion (maintained in Eagle's medium with 4% horse serum). It also propagated in monkey testicular and bovine kidney cell cultures as evidenced by development of hemadsorption after a period of negativity (Table II). Hemadsorption test may be of considerable advantage in rapid laboratory diagnosis of mumps.

Croup-Associated (CA) Virus. A MK passaged prototype strain (Greer) produced cytopathic effects and hemadsorption with guinea pig cells in MK, human amnion, and HeLa cells. Five cytopathogenic viruses isolated in MK cultures from throat swabs collected from patients with undifferentiated respiratory illness in 1955, were identified as CA virus by hemadsorption-inhibition (using normal, mumps, Sendai, and CA guinea pig antiserums). Hemadsorption reaction appears to be convenient and reliable method for identification of this virus.

Other pathogenic viruses.‡ Prototype strains of other viruses pathogenic for human beings were tested for hemadsorption in appropriate TC systems and those belonging to

‡ Exploratory experiments at Rockefeller Foundation Virus Laboratories suggest that hemadsorption may occur with certain arthropod-borne viruses.

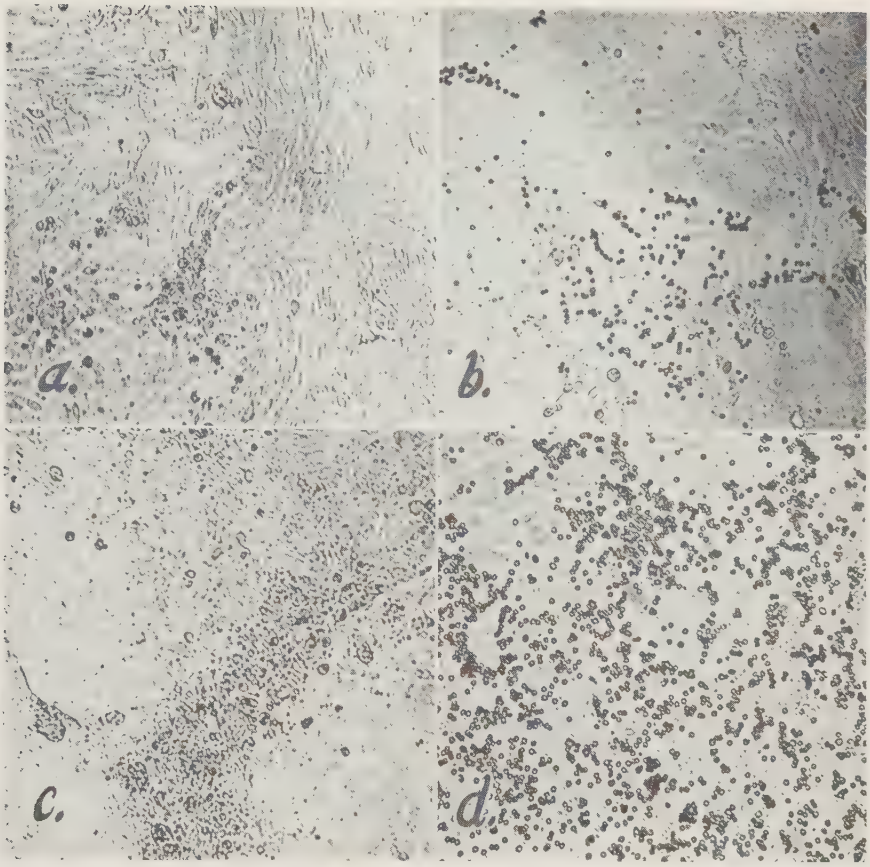


FIG. 3. Mumps virus in MK culture. Hemadsorption with guinea pig erythrocytes at 48 and 72 hr (×105). *a.* 48 hr: no CPE. *b.* Same with erythrocytes added. *c.* 72 hr: probable CPE. *d.* Same with erythrocytes added.

hemagglutinating group were generally positive. Table I summarizes origin and history of these strains. *Sendai.* Two strains from different sources after passage in MK produced hemadsorption with both guinea pig and chicken cells. *Newcastle disease virus (NDV).* An egg-adapted strain was propagated in chick embryo monolayer cultures as shown by hemadsorption with chicken and guinea pig erythrocytes for each of 5 serial passages. *Vaccinia.* MK adapted vaccinia strain produced heavy hemadsorption with erythrocytes from 2 individual chickens but not with guinea pig or sheep cells. The virus was propagated for 5 passages in chick embryo TC with invariably positive hemadsorption with chicken cells from the same 2 birds. *Encephalomyocarditis* (EMC). No viral propagation could be demonstrated upon repeated inoculation of EMC-infected mouse brain into MK and chick embryo TC. Hemadsorption was always absent, although on addition of brain inoculum suggestive clumping of sheep erythrocytes occurred in virus-laden nutrient fluid. This was not observed with guinea pig or chicken cells. *Other prototype virus strains propagated in MK cultures.* Poliovirus types 1, 2, 3, and herpes simplex virus propagated in MK cell cultures failed to cause hemadsorption with guinea pig, chicken, and sheep red cells. Coxsackie A9, B1 through B5, and ECHO virus types 1 through 11, were also negative with guinea pig and human erythrocytes. Attempts with adenovirus types 1, 2, 4, 6, 7, and 8 were equally unsuccessful with guinea pig and sheep cells. *Simian viruses.* Occasionally, upon incubation of certain batches of uninfected control tubes with added erythrocytes, we found that red cells adhered to the monolayer MK cell

sheet. Because characteristic patterns of tight clusters, rosettes, and streamers were missing, we named this appearance *sticking* of erythrocytes to distinguish it from the typical pattern of hemadsorption. Sticking seemed to be particularly troublesome with certain lots of trypsinized MK cells and we suspected it to be due to simian orphan viruses. To elucidate this problem we studied the behavior of Hull's virus types SV 2, 4, 5, 12, and 15 reported by him to cause hemagglutination under variable conditions(7). We repeatedly passaged prototype strains with production of characteristic CPE and regularly checked the cultures for presence, intensity, and character of reactions with guinea pig, chicken, human O, and rhesus monkey erythrocytes. Our results with type SV4 were consistently negative regardless of species of erythrocytes used or conditions provided. The other prototype strains gave erratic results on many trials, ranging from negative to reactions resembling familiar hemadsorption patterns. The results apparently depended on such factors as incubation temperature (4°, 22°, 37°), whether readings were taken after a few minutes, several hours, or overnight contact of erythrocytes with the TC sheet, and whether contact was constant in a stationary rack or intermittent in revolving drum. Adherence of erythrocytes to MK cells caused by hemagglutinating simian viruses and the sticking observed in our uninoculated cultures were distinguishable from what we described as hemadsorption. However, it is possible that some simian viruses pre-existing in MK cells might produce the same type of pattern as with other hemagglutinating viruses.

Use of hemadsorption reaction in serologic studies. Paired serums from 4 patients with known 8 to 16-fold rise of hemagglutination-inhibiting antibodies against Asian strain of influenza virus were heated at 56°C for 30 minutes, but were not subjected to other special treatment to remove inhibitors. Serial serum dilutions were tested by neutralization-hemadsorption test against approximately 500 $TCID_{50}$ of a TC adapted cytopathogenic strain of Asian influenza virus. Demonstrable antibody rise as measured by prevention of eventual hemadsorption was similar to that shown by standard hemagglutination-inhibition test. The hemadsorption-inhibition procedure, which we preferred for typing isolates with potent immune animal serums, was less satisfactory because repeated checking of virus-inoculated control tubes for hemadsorption was necessary to determine optimal time for performing inhibition test. When incubation proceeded to a point of recognizable CPE excessive amounts of virus were usually present.

Discussion. Demonstration of specific erythrocyte adsorption by several important hemagglutinating viruses propagated in TC systems provides a practical approach to diagnostic and fundamental studies of these agents. Because hemadsorption depends on direct visualization, it is detectable in presence of minimal hemagglutinins associated with TC cells, which results in greater sensitivity than with procedures based on development of CPE or the usual hemagglutination by virus suspensions from TC supernates or egg fluids. The technic was simple, rapid, and specific. Hemadsorption reaction is a useful technic for identification not only of viruses which produce otherwise undetectable hemagglutinins in TC fluids but also of agents which may propagate without apparent damage to TC cells. Undoubtedly it will find application in fundamental studies of virus-cell relationship.

Our continuing experience with this reaction prompted us to adopt the name of *hemadsorption* in place of *adsorption-hemagglutination* proposed initially(1). Indeed, even though the phenomenon undoubtedly is due to hemagglutinin production within infected cell, the particular practical advantages of this reaction are due to selective erythrocyte adsorption onto monolayer surface of cultured tissue cells rather than three-dimensional clumping of erythrocytes, that is, true hemagglutination.

Summary. The phenomenon of hemadsorption is dependent upon selective attachment of erythrocytes onto the monolayer surface of tissue culture cells. It is demonstrated by addition of erythrocytes to a tissue culture system in which propagation of a hemagglutinin-producing virus has occurred. We used

this reaction as a means of virus detection in isolation, titration, and identification of influenza A, mumps, and CA (croup-associated) viruses. Prototype strains of 2 other myxoviruses (Sendai and NDV) and vaccinia produced hemadsorption in appropriate cell cultures. Prototype strains of common nonhemagglutinating viruses (herpes, Coxsackie, ECHO, adeno- and polio-viruses) gave negative results. Under some conditions hemagglutinating simian agents, which may be encountered in monkey kidney cell cultures, caused adherence of erythrocytes to the tissue cell sheet, but the characteristic patterns seen with myxoviruses were not observed. We found hemadsorption to be a simple, direct, and reliable test offering a new approach to diagnosis of some viral infections. It may also provide an investigative tool for research on virus-cell relationships.

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Influence of Female Sex Hormones in Experimental Teratogenesis.* (23885)

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Studies of congenital malformations deserve increased emphasis because of their importance as a significant health problem. Many investigators have demonstrated experimental production of mammalian congenital defects by alteration of maternal environment. The methods have included: nutritional deficiences(1-4), vitamin deficiencies (5-7), radiation(8-10), hypoxia(11-12), and more recently, cortisone(13-15). Present data indicate that under certain conditions estrogens are also teratogenic.

Materials and methods. Virgin female mice of the Webster-Swiss strain, weighing around 25 g, were employed. They were kept at least 2 weeks after shipment, and used after reaching the indicated weight. They were fed Rockland mouse diet in pellet form.‡ We employed 2 series: (A) Estrone ("Theelin") in peanut oil (1 mg/cc); and (B) Estradiol Benzoate in sesame oil (1 mg/cc). As a control for group (1), nothing was given to a comparable number of mice; and for group (2), a similar volume of sesame oil was administered. Injections were given between 10:00 and 11:00 a.m., except those on eleventh day of gestation, done at 5:00 p.m. During administration No. 22 gauge needles were inserted subcutaneously from median part of back into subcutaneous layer of left lateral breast region. This eliminated any spillage of injected material through the puncture wound. Preliminary experiments were performed upon a large number of mice to decide upon a proper dose of estrone and estradiol benzoate. 0.2 mg daily was the dosage level at which the incidence of cleft palate was highest, and the

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‡ Rockland Farms, New City, N. Y.

TABLE I. Comparison of Effect by Estrone and Estradiol Benzoate.

	No. of mothers sectioned	No. intact fetuses obtained	No. of cleft palate				% of cleft palate	No. of absorbed fetuses	Absorbed fetuses / Intact fetuses
			Total	(L)	(M)	(S)			
							%		%
Estrone	18	81	10	3	4	3	12.4	69	85.2
Estradiol benzoate	13	81	11	4	4	3	13.6	29	35.8
Sesame oil inj. cont.	20	143	1		1		.7	23	16.1
Untreated control	14	115	0				0	11	9.6
Unsectioned, untreated control	13	95	1	1			1.1	unknown	

L = large cleft palate; M = medium cleft palate; S = small cleft palate (see Fig.).

number of abortions lowest. Day of appearance of vaginal copulation plug was counted as first day of gestation. Mice with plugs were separated, and the animals with bonafide pregnancies sorted by palpation on eleventh day following initial isolation. Eleventh through sixteenth days of gestation were chosen for injections since it is known that the mouse palate closes between the fourteenth and fifteenth day. To prevent loss of offspring through cannibalism, uterine contents were removed by section on eighteenth day.

Results. In Table I, the comparative effects of estrone and estradiol are shown, with 0.2 mg of each given on twelfth and fifteenth day, and 0.1 mg on thirteenth, fourteenth and sixteenth day. It is evident that there was no difference in rate of cleft palate formation induced by estrone and estradiol benzoate. It is possible that there is a correlation between fetal absorption and cleft palate which could alter this relationship (see discussion of Table II below) if a dosage change resulted in more intact fetuses.

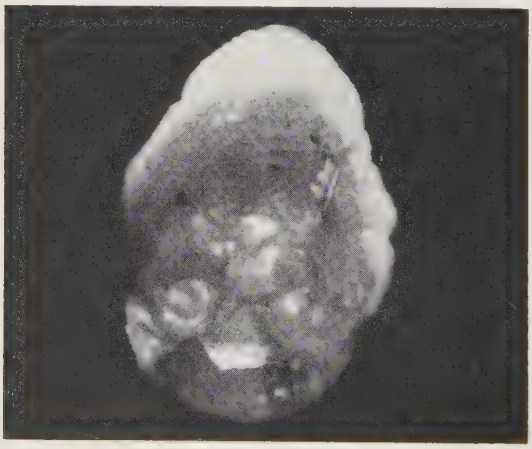


FIG. 1. A normal palate.

It should be noted that doubtful clefts appeared in both groups in which the palates were in various stage of closure. These were counted as normal (Fig. 3). Further attempts (with estrone) were made to increase the estrogen effect upon production of congenital cleft palate. In these experiments, Group A was given 0.2 mg on the 12th and 15th day, 0.1 mg on 13th, 14th and 16th day, and additional 0.05 mg on 17th day. This additional injection was given empirically, because premature deliveries were occasionally noted at the usual time of section on 18th day. The additional dose apparently prevented this difficulty. To Group D, 0.2 mg was administered on the 11th and 14th day, and 0.1 mg on 13th, 15th and 16th days, with additional 0.005 mg on 17th. In Group C and D, nothing was given on the 12th day, but injections were begun on 11th day, since the estrone was in an oil base, and it was therefore considered wise to begin one day earlier. Finally, in Group E, 0.2 mg estrone was given once a day, starting from the 11th. The results are summarized in Table II.

If, under these experimental circumstances, there were an increase in the cleft palate rate in the group receiving most estrogen, one could reasonably conclude that there is a relationship between estrogen level and propensity to produce defective offspring.

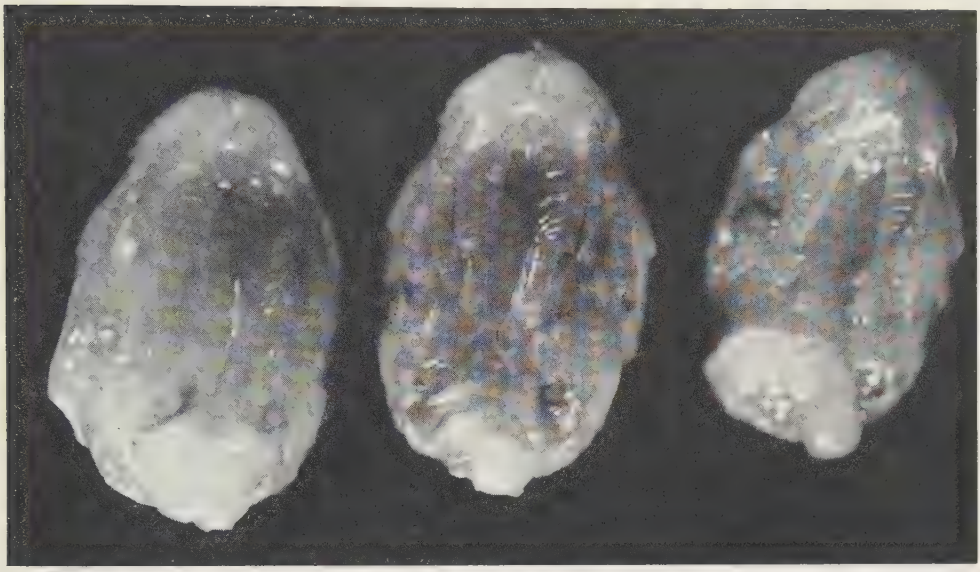


FIG. 2. Estrogen-induced cleft palates. From left to right: small, medium, and large defects.



FIG. 3. Two examples of doubtful clefts. These exhibited a thin palate at the line of closure and a depression along the symphysis. Recorded as normal in results.

It should be noted, however, that even in Group D, 2 mothers out of 15 did not produce cleft palate offspring. In Group E, 6 pregnancies out of 10 ended in absorption of fetuses. Since it appeared difficult to obtain sufficient data for valid comparison with other groups, this constant dose experiment was not carried further. However, this group suggests that if estrogen is maintained at a plateau level, the fetuses are apt to be absorbed rather than coming to term with cleft palates.

Discussion. These data demonstrate that parenteral estrogen injection has teratogenic effects on developing mice embryos when administered to mothers under the stated experimental conditions. These effects are not so frequent as those demonstrated by Fraser, whose technic of cortisone administration§ has resulted in a 90% incidence of cleft palate formation in our hands. They are definite, however; and they indicate that an additional emphasis is needed on the role of estrogen secretory fluctuations in the pathogenesis of human cleft palates.

Conclusion. Under the conditions of this experiment, estrogen acted as a teratogenic agent.

§ 2.5 mg of cortisone acetate/day for 4 days starting on 12th day of gestation.

TABLE II. Comparison of Estrone Effect by Groups.

	No. of mothers sectioned	No. intact fetuses obtained	No. of cleft palate				% of cleft palate	No. of absorbed fetuses	Absorbed fetuses / Intact fetuses
			Total	(L)	(M)	(S)			
							%		%
Group A	18	81	10	3	4	3	12.3	69	85.2
B	15	88	13	3	8	2	14.8	36	40.9
C	19	63	17	5	7	5	27.0	72	114.3
D	15	71	25	10	6	9	35.2	43	60.6
E	10	14	3			3	21.5	62	442.9

This work was done in the laboratory of Dr. John F. Prudden. His advice and encouragement are gratefully acknowledged.

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Desoxyribonuclease (DNA'ase) Activity of Rat Mammary Gland During Pregnancy and Lactation.*† (23886)

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In previous publications(1,2), it was reported the content of desoxyribonucleic acid (DNA) of rat mammary gland epithelial cells decreased following parturition(1); and DNA-phosphorous of mammary gland also declined at day of parturition when determined on dry tissue(2). An explanation of these unusual findings was sought in determining DNA'ase activity of mammary gland during pregnancy and lactation to see if there was correlation between DNA and DNA'ase activity.

Methods. Data were obtained from primiparous albino rats during late pregnancy (18-20 days), day of parturition and during advanced lactation (20-21 day). The animals were killed, the 6 posterior mammary glands rapidly removed. These were placed in ice-cold sucrose or saline solution, diluted to 50 mg/ml, homogenized for 2½ minutes in a Waring Blendor, then centrifuged for 10 minutes at 2600 rpm. The supernatant was saved for the following procedure. Six individual samples were prepared by addition of one ml

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TABLE I. DNA'ase Activity of Mammary Gland in Late Pregnancy and Early and Late Lactation.

	No. of animals	$A_2 - B_2$*	$B_1 - A_1$†
Pregnancy	12	Mean 277 ± 30.98	307 ± 30.78
Early lactation	11	Mean 588 ± 53.30	−47.3 ± 124.45
Late lactation	12	Mean 377 ± 32.65	345 ± 35.75

* $A_2 - B_2$ = decrease in DNA after 1 hr incubation in μg.

† $B_1 - A_1$ = increase in acid-soluble nucleotides after incubation in μg.

of supernatant to substrate solution containing 2400 μg of DNA/ml and one ml of acetic acid, sodium acetate buffer(3,4). Three samples were used as controls to determine DNA (A_2 fraction) and level of acid-soluble nucleotides (A_1 fraction) before incubation. This was accomplished by addition of 5 ml of 10% TCA to 1 ml of supernatant. Remaining 3 samples were placed in water bath for one hour at 37°C, centrifuged and resulting precipitates separated from supernatant to determine DNA (B_2 fraction) and acid-soluble fraction (B_1 fraction). DNA'ase activity was estimated by decrease in DNA substrate as compared with control samples, or by increase in acid-soluble fraction as compared with control samples. Each method acted as a check on the other, a decrease in DNA causing a corresponding increase in the acid-soluble fraction. A_2–B_2 = decrease in DNA, B_1–A_1 = increase in acid-soluble nucleotides. Preliminary observations indicated enzyme activity of the complete homogenate demonstrated wide variance between enzyme activity and nucleotides, probably caused by unequal distribution of nuclei in the aliquots. The method of Webb and Levy(5) was used to determine DNA and acid-soluble fractions.

Results. DNA'ase activity of mammary glands in late pregnancy was lower than in either early or late lactation, while decrease in DNA during pregnancy was directly correlated with increase in acid-soluble nucleotides (Table I). The average decrease in DNA was 277 μg released/hour, while the increase in the acid-soluble fraction was 307 μg/hour. On the first day of lactation there was an abrupt increase of over 100% in enzyme activity and a marked decrease in acid-soluble fraction as compared with late pregnancy (B_1). There was a marked decrease over pregnancy. In half the cases this decrease ranged from −195 μg to −570 μg below controls, while in remaining fractions increase in acid-soluble nucleotides was not proportional to the decrease in DNA. Average enzyme activity for this stage of lactation was 588 μg/hour; the average increase in acid-soluble nucleotides was −47.3 μg/hour. By 20th day of lactation enzyme activity had decreased to average of 377 μg/hour and the increase in the acid-soluble fraction was again proportional to the decrease in DNA with average increase of 345 μg/hour.

There was a significant difference in DNA'ase activity in both pregnancy and late lactation as compared with early lactation ($P < .0025$). The difference between pregnancy and late lactation approached significance ($P > .05$).

Discussion. During pregnancy and late lactation enzyme activity is directly correlated with an increase in acid-soluble nucleotides. During these 2 periods DNA/cell remains within normal values. Increase in DNA'ase activity coupled with decrease in acid-soluble nucleotides on first day of lactation suggests that the decrease in DNA/cell is due to greater catabolism of cellular components reflecting increased need by animal for sugar and phosphate during milk secretion. DNA'ase activity was not compared on basis of protein nitrogen or dry weight during late pregnancy or lactation because the mammary gland is actively synthesizing milk, thus variable amounts of protein, fat and water would be present during these periods.

Summary. DNA'ase activity was determined in late pregnancy and in early and late lactation in conjunction with measurement of acid-soluble nucleotides. DNA'ase activity was correlated with increased acid-soluble nucleotides during pregnancy and latter stages of lactation. There was, however, on first day of lactation an increase in enzyme activity and a decrease in acid-soluble nucleotides suggesting an increased catabolism of these products in early lactation for use in secretory process.

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Effects of Saturated and Unsaturated Fat on Cholesterol Metabolism in the Rat. (23887)

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Studies in several laboratories have established the remarkable effects of unsaturated fats in lowering serum cholesterol levels in man, *e.g.*(1,2). The mechanism by which these fats act is not yet known. While effects in animals may be different in some respects from those in humans, the extra parameters that can be evaluated make animal studies worthy of exploration. Previous animal studies on effects of dietary fat have frequently been complicated by simultaneous feeding of cholesterol, of cholesterol-containing animal fat, or other atherogenic factors. In our studies a comparison is made of effects of 2 vegetable oils, corn oil and coconut oil, on rate of cholesterol biosynthesis and on distribution of cholesterol in body pools in rats on a cholesterol-free diet.

Methods. Adult male rats of Osborne-Mendel strain were fed *ad libitum* with ground Purina chow containing 20% by weight of corn oil or coconut oil. Both oil-containing diets were consumed at about same rate, supplying animals with same amount of calories. The rats given these diets had a caloric intake higher by about 35% than control rats on Purina chow alone. At an exactly defined time before sacrifice, animals were injected intraperitoneally with a tracer amount of labeled compound serving as cholesterol precursor. The rats were anesthetized with ether, killed by bleeding and livers were removed and chilled. Aliquots of liver, serum or whole adrenal glands were extracted with alcohol-acetone 1:1, saponified and the non-saponifiable fraction counted in the Packard scintillation counter. Free and esterified cholesterol in tissues were determined according to Sperry and Webb(3).

Results. Table I shows effect of diet on cholesterol levels in serum. Except in Exp. I, the shortest one, both coconut oil and corn oil fed rats had higher serum cholesterol levels than controls. Comparison of corn oil fed animals with coconut oil fed animals shows that in all experiments, irrespective of feeding period, total serum cholesterol of corn oil fed rats was lower. In 2 experiments the P value of this difference was between 0.05 and 0.1. However, the difference was never very large compared with that observed by others in man. Livers from these animals were analyzed for cholesterol content and here the effects of diet were marked (Table II). Cholesterol concentration in liver of corn oil fed animals was almost twice that in controls. The effect of feeding coconut oil was much smaller and not always significant. Of particular interest was the fact that almost all the increase was in the normally small cholesterol ester fraction. There were only small differences between groups in concentration of free cholesterol.

TABLE I. Effect of Diet on Cholesterol Concentration of Rat Serum.*

Exp.	Duration of feeding (days)	Serum cholesterol (mg %) Control diet	Coconut oil diet	Corn oil diet
I	17	68.5 ± 6.9†	77.6 ± 13.6	63.4 ± 3.2
II	20	75.1 ± 7.9	87.0 ± 10.0	82.4 ± 10.7
III	32	68.8	79.9	76.6
IV	80	60.1 ± 6.3	81.1 ± 10.9	67.7 ± 8.9

* Each figure represents mean value for 6 rats.
† ± stand. dev.

TABLE II. Effect of Diet on Cholesterol Content of Rat Liver.

Exp.		Cholesterol concentration (mg/g wet tissue) Control diet	Coconut oil diet	Corn oil diet
I	Free cholesterol	2.29	2.04	2.53
	Esterified "	.07	.57	1.93
II	Free "	2.03	1.90	2.28
	Esterified "	.28	.46	1.45
IV	Free "	1.94	1.89	2.24
	Esterified "	.11	.16	1.59

TABLE III. Effect of Sterol-Free Linoleic Acid on Liver Cholesterol Levels.*

	Linoleic acid diet	Linoleic acid diet + sterols
Liver free cholesterol (mg/g)	2.11	2.05
" esterified " (")	1.37	1.01
Serum total " (mg/%)	72.7	73.1

* Animals were on respective diets 20 days. Each figure represents mean value for 6 rats.

We next investigated the role of vegetable sterols in changes brought about by corn oil feeding. One group of rats received a diet containing sterol-free linoleic acid preparation. A second group received linoleic acid in same amount together with mixed sterols prepared from commercial corn oil. The results shown in Table III clearly indicate that, like crude corn oil, purified linoleic acid itself leads to accumulation of esterified cholesterol. This finding rules out the possibility that the sterol appearing in liver is to any significant extent contributed by vegetable sterols in oil. Addition of sterols did not significantly increase the amount of cholesterol accumulating in liver nor did it affect total serum cholesterol. This strongly suggests that unsaturated fatty acids rather than sterols in corn oil are responsible for changes in lipid composition of liver.

Rate of *in vivo* hepatic synthesis of cholesterol from 1-C^{14}-acetate was compared in rats under various dietary conditions. The results are given in the first 2 lines of Table IV, both as total counts/g of liver and as specific radioactivity. Coconut oil feeding did not influence total counts incorporated but corn oil increased it significantly. Since there was a higher concentration of cholesterol in livers of corn oil fed animals the specific radioactivity of cholesterol remained the same as in the 2 other groups. The possibility had to be considered that different dietary fats might be metabolized at different rates, affecting to a different degree the size of pools of 2 carbon fragments, which serve as precursors of the cholesterol molecule. To eliminate this possible effect of diet on rate of incorporation of labeled acetate, the study was repeated using tritiated water as the cholesterol precursor. As seen in Table IV, there was again an increase in total count incorporation in corn oil-fed animals.

Rate of isotope incorporation into cholesterol of serum and of adrenals is shown in Table V. In serum both total non-saponifiable lipid counts and specific radioactivity of cholesterol were markedly higher in corn oil-fed rats than in the other 2 groups. Total count incorporation from T_2O into adrenal non-saponifiable lipid was also markedly increased in corn oil-fed rats.

Distribution of cholesterol in cell fractions was also investigated. In cell-free liver homogenates of oil-fed rats there was a marked increase in a fat-rich fraction floating at density 1.006 on ultracentrifugation. Most of the excess cholesterol over amount found in control-fed animals was present in this layer. This "free fat" fraction increased also after cholesterol feeding as reported by other authors(4).

TABLE IV. Incorporation of 1-C^{14}-acetate and T_2O into Nonsaponifiable Lipids of Rat Liver.

Exp.	Precursor	Non-saponifiable lipid radioactivity (cpm/g liver) Control	Coconut	Corn	Cholesterol spec. radioactivity (cpm/mg) Control diet	Coconut oil diet	Corn oil diet
I	1-C^{14}-acetate	1524 ± 480*	1478 ± 848	3528 ± 1378	643	584	685
IV	"	438 ± 204	389 ± 86	693 ± 346	218	192	183
II	T_2O	161 ± 38	181 ± 97	225 ± 98	88	92	78

* ± stand. dev.

TABLE V. Incorporation of 1-C^{14}-acetate and T_2O into Nonsaponifiable Lipids of Rat Serum and Adrenals.

Tissue	Exp.	Precursor	Non-saponifiable lipid radioactivity — Control	Coconut	Corn	Cholesterol spec. radioactivity (cpm/mg) — Control diet	Coconut oil diet	Corn oil diet
			(cpm/ml)					
Serum	I	1-C^{14}-acetate	152 ± 33*	179 ± 66	266 ± 72	220	250	416
			(cpm/mg tissue)					
Adrenals	II	T_2O	1.33 ± .45	1.02 ± .41	2.72 ± 1.44			

* ± stand. dev.

Discussion. Our results clearly indicate that feeding rats corn oil, or linoleic acid, the major component of corn oil, increases markedly the *liver* cholesterol pool. At the same time the serum cholesterol is to a small, but significant extent lower than in rats receiving coconut oil. Both oil-fed groups have higher serum cholesterol levels than controls. The increased accumulation of cholesterol in livers of rats fed with unsaturated oils was also observed by Grunbaum *et al.*(5), who used sunflower oil, and Brown and Lewis(6), for soybean oil. The latter authors also report some tendency for lower serum cholesterol where the hepatic cholesterol was highest.

The present data also show that on feeding corn oil, the overall rate of C^{14}-labeled acetate or T_2O incorporation increases, which probably represents an elevated net synthesis/unit time. If a steady state is prevailing, and this is probably a fair assumption in long term experiments, the rate of excretion or breakdown of cholesterol must also be increased in corn-oil fed animals. Recent studies by Hellman and coworkers suggest that this is the case in man(7). The finding of an increased rate of incorporation of isotopic precursors into non-saponifiable lipids in livers rich in cholesterol seems to contradict the prevailing theory of homeostatic control of cholesterol synthesis in this organ. One should note, however, that in livers of cholesterol-fed rats the concentration of free cholesterol is markedly increased over the controls(4), whereas in corn oil-fed animals the amount of this fraction is only very slightly elevated (Table II). On feeding corn oil the size of cholesterol pool in rats increases but at the same time the partition within this pool between plasma and liver is affected by some unknown mechanism in such a way that there is less cholesterol in blood. If qualitatively a similar situation exists in man one should, perhaps, look at the change in partition of cholesterol between the 2 compartments as a key to understanding of the effect of unsaturated fats on serum cholesterol. Information on this point would certainly be of importance in any final evaluation of dietary therapy of hypercholesterolemia.

Summary. 1. Rats fed a diet containing 20% corn oil have significantly lower concentrations of serum cholesterol than those fed equal amounts of coconut oil but both high-fat diets lead to elevation of serum cholesterol above that seen on control diets. 2. There is a very marked increase in esterified cholesterol of livers of rats fed corn oil whereas coconut oil-fed rats show no significant changes in liver cholesterol compared to controls. 3. Rate of incorporation of 1-C^{14}-acetate or of T_2O is higher in corn oil-fed rats than in others. 4. Some implications with respect to the mechanism by which unsaturated fats alter cholesterol metabolism are discussed.

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Determination of Calcium in Biologic Material by the Chloranilate Method. (23888)

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Calcium is precipitated by chloranilic acid, a reagent which is red-purple. Teeri(1) applied this reaction in determining calcium in serum, first separating calcium as the oxalate, then dissolving the oxalate with acid and determining calcium by photometrically measuring decrease in color of chloranilate added in excess. Ferro and Ham(2,3) approached the analysis in a different way, precipitating calcium from serum as chloranilate, then dissolving the latter in a solution of ethylenediamine tetraacetate at high pH and determining color of liberated chloranilate photometrically. In comparing the method of Ferro and Ham with that of Clark and Collip(4), the present authors experienced difficulty with the former method because of turbidity in the final colored solution in a significant number of samples. This communication deals with a solution to this problem, applies the method to determination of calcium in urine and feces and gives comparative results obtained by chloranilate and Clark-Collip methods. An ultramicro adaptation of the method to serum is also presented.

Methods. Reagents. 1. Chloranilic acid reagent. Dissolve 4 g of NaOH pellets in about 650 ml distilled water in a liter volumetric flask. Add 11 g of chloranilic acid (E.K. No. P 4539) and make up to 1 liter with water. Agitate until acid dissolves. The pH should be less than 11. Usually the pH is about 7 but, if greater than 11, additional 1 g quantities of chloranilic acid are added to reduce the pH. Filter through a Whatman No. 1 paper using a Buchner funnel. Filter again through a Seitz pad, No. ST-1 or ST-3 (Hercules Filter Corp., Size L-6). The reagent is stable in refrigerator for about 6 months but only 2 to 3 months at room temperature(3). 2. Tetrasodium ethylenediamine tetraacetate, 5% (EDTA reagent). Ferro and Ham employed tetrasodium salt but, since this is difficult to obtain, the required reagent can be prepared from disodium ethylenediamine tetraacetate dihydrate (Baker, A. R. grade) as follows: Add 44.8 g of disodium salt to about 800 ml of water in a liter volumetric flask, dissolve the salt by adding 10% NaOH drop by drop until pH 10.3 is reached, then add water to 1 liter. 3. Isopropyl alcohol, 50% (V/V). 4. Calcium standard, 200 μg/ml. Weigh exactly 500 mg Iceland Spar and transfer to a 1-liter volumetric flask. Add 14 ml dilute HCl (1 part conc acid + 9 parts distilled water). Let stand until solid is dissolved, then add distilled water to 1 liter. *Ultramicro procedure for serum.* Set up the following in 2-ml conical-tipped centrifuge tubes: *Blank.* 100 μl water. *Standard.* 100 μl calcium standard containing 20 μg of calcium. *Serum.* 100 μl of serum. To each tube add 100 μl of chloranilic acid reagent, mix and let stand 2 hours. (Contrary to the claim(3) that 30 minutes are sufficient for complete precipitation of calcium chloranilate from serum samples, the present authors found that 2 hours were required as a safe minimum.) Centrifuge and remove supernate either by carefully decanting or by siphoning with reversed tipped capillary. Invert and drain 3 minutes, then wipe mouths of tubes with absorbent tissue. Wash precipitate with 0.15 ml of 50% isopropyl alcohol, stirring precipitate with a rod. Wash rod with 0.1 ml wash reagent. Centrifuge, discard supernate, invert and drain. Repeat washing procedure. Dissolve washed precipitate in 0.3 ml EDTA reagent, stirring with glass rod. Centrifuge. Examine unknown for turbidity by checking for Tyndall effect. If turbid, extract with 0.15 ml ethyl ether. Centrifuge and read absorbances of clear, aqueous phases against distilled water at 525 mμ, the absorbance peak, in Bessey-Lowry micro cuvets (Pyrocell Mfg. Co., N. Y. 28) in Beckman Model DU spectrophotometer. Occasionally, in an extremely lipemic serum, it may be necessary to extract with 2 aliquots of

ether for clarity. Calculation: $\frac{A_X - A_B}{A_S - A_B} \times 20 =$ mg Ca/100 ml serum. *Macro procedure for serum.* Similar to ultramicro procedure except that 2 ml quantities of distilled water, standard and unknown are placed in 12-ml, heavy wall centrifuge tubes and 1 ml chloranilic acid reagent added. Precipitates are washed with 5 ml isopropyl alcohol and finally dissolved in 6 ml EDTA reagent. If turbid, extract with about 3 ml of diethyl ether. Absorbances can be read in a spectrophotometer at 525 mμ or with a filter with nominal wavelength in this region (Beer's law was followed at 525 mμ on Beckman Model DU spectrophotometer and on a Klett photometer with a No. 54 filter). Calculations are the same as for ultramicro procedure. *Procedure for urine.* Transfer 10.0 ml of well-mixed urine to a test tube. Acidify to at least pH 1 by adding several drops of conc. HCl and testing with pH paper. Mix, mark meniscus on the tube and place in boiling water for about 30 minutes. Cool to room temperature, add water to mark, mix and centrifuge. Pipet 2.0 ml of supernate to a 12-ml, thick-walled centrifuge tube, adjust pH to 4.5 with 2% NH_4OH using narrow range pH paper, add 1.0 ml chloranilic acid reagent and proceed and calculate as for serum. *Procedure for feces.* Weigh approximately 1 g of feces into a small porcelain crucible. Dry sample on a steam bath. Ash with gentle flame for a few minutes until most of the carbon is burned off. Cool and add a few drops of water. Mix and break up the remaining carbon with a stirring rod. Dry on the steam bath. Ash again until no carbon remains. Cool and add 3 drops of 6 N HCl to dissolve the ash. Dry again on the steam bath. Add 2 more drops of 6 N HCl and transfer quantitatively with hot distilled water into a 12-ml, thick-walled, graduated centrifuge tube. Add water to 10.0 ml and mix. Transfer 1.0 ml to another centrifuge tube, add 1 ml of water, adjust the pH to 4.5 with 2% NH_4OH using narrow range pH paper. Add 1.0 ml chloranilic acid reagent and proceed as for serum. Calculation:

$$\frac{A_X - A_B}{A_S - A_B} \times \text{mg Ca in standard} \times \frac{1000}{\text{g wet weight of feces in sample}}$$

= mg Ca/100 g wet weight of feces. *Clark-Collip method.* The method of Clark and Collip(4) was followed for serum except that the calcium oxalate was quantitated by perchloratoceric acid titration using ferroin as indicator(5). For urine and feces the method was applied to ashed specimens.

TABLE I. Interference by Turbidity.

Clark-Collip, mg Ca/100 ml	Chloranilate: Unextracted, mg Ca/100 ml	Chloranilate: Unextracted, Turbidity	Chloranilate: Extracted, mg Ca/100 ml
9.0	9.1	0	9.1
10.1	9.6	0	9.6
9.1	9.4	1+	9.2
9.4	10.0	2+	9.5
9.5	12.4	3+	9.6
9.9	13.2	3+	9.7
9.1	11.6	4+	9.0

Results. Interference in analysis of serum by turbidity. Table I gives representative comparisons between results (means of duplicate analyses) obtained by the Clark-Collip method and the chloranilate method, with and without ether extraction. That the turbidity is lipid in nature is indicated by the facts that it was removed by ether as well as other lipid solvents and that, in general, the degree of turbidity increased with the lipid content of the sample (*e.g.*, samples 5-7 in Table I were grossly lipemic). The appearance of turbidity at the final stage could be reduced but not completely avoided by multiple washings of the calcium chloranilate precipitate with the isopropyl alcohol wash reagent. Because of the volume change of the aqueous phase when saturated with ether, the absorbance of standards and unknowns decreases with ether extraction. The mean decrease in absorbance of standards and clear unknowns was 5%. Thus, rather than reading extracted unknowns against extracted blanks and standards, a correction can be made. It is seen from Table I that gross positive errors can occur if interference by turbidity is not avoided. Turbidity was never observed in this study with urine or fecal samples.

Comparison of results with the method of Clark and Collip. Nineteen samples of serum were analyzed in duplicate by both methods. The results by the chloranilate method averaged 0.1 mg/100 ml greater than those ob-

tained by the Clark-Collip procedure but this bias was not significant at $p = 0.05$ by the t test(6). In a similar study of 21 urines, results by the chloranilate method averaged 1.3 mg/100 ml greater than those yielded by the Clark-Collip procedure. This difference was significant at $p = 0.05$ but not at $p = 0.01$ (t test). Similarly, results of fecal calcium were about 5 to 10% higher by the chloranilate method. The cause for these discrepancies is not apparent and it is impossible at this time to tell which method is more accurate. For most purposes, however, this difference is unimportant.

Reproducibility. The standard deviations (mg/100 ml), calculated from duplicate analyses, were as follows: 0.16 and 0.59 for the Clark-Collip method applied to serum and urine, respectively; 0.17 and 0.16 for the chloranilate method applied to serum and urine, respectively. This means that by the chloranilate method the precision (95% limits) of a single analysis of serum calcium was about $\pm$ 3.2% and that for a single analysis of urine calcium at a level of 25 mg/100 ml was about $\pm$ 1.5%.

Discussion. It is the authors' opinion that the chief advantage of the chloranilate method for the determination of serum calcium is the working time saved when many samples are to be run. The advantage of direct application to urine without preliminary ashing is obvious. The precision of the technic for both serum and urine is quite good and the results, in the case of serum, appear identical to those obtained by the Clark-Collip procedure. With urine and fecal samples, the results by the chloranilate method are slightly higher than those obtained by the Clark-Collip procedure.

Summary. The Ferro-Ham chloranilate procedure for determination of serum calcium has been investigated. Modifications of this procedure are presented for determination of urine and fecal calcium and ultramicro modification for determination of serum calcium is described. Presence of turbidity, apparently lipid in nature, in final solution constitutes a source of significant positive error in the chloranilate method for determination of calcium in serum. This turbidity can be removed by ether extraction. Results obtained by the chloranilate method have been compared with the Clark-Collip procedure.

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Effect of Some Hormones on Calcium Balance in Elderly Subjects.* (23889)

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Our earlier studies on calcium and phosphorus balance in elderly subjects have shown that calcium requirements are higher for such subjects than other workers found for young adults, with little change in phosphorus requirements(1,2). We later noted that addition of Vit. D to a supposedly adequate diet increased calcium retention in these subjects (3). Increased calcium requirements in the aged make it more likely that such subjects will be in negative calcium balance on an ordinary diet. The increase in incidence of osteoporosis in the aged has been noted by many workers(4). We have been studying the ef-

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fects of various hormones on nitrogen balance in elderly subjects for some time. Some cause an increase in nitrogen retention and definite clinical improvement(5,6). Use of these hormones has found a definite place in treatment of debilitated elderly subjects(7). In view of the precarious state of calcium equilibrium in such individuals, we felt it advisable to make further studies on the effect of these hormones on calcium balance, as any effect in decreasing calcium retention would be undesirable.

We present results on effect of thyroid, insulin, cortisone and ACTH on calcium balance. We also give a revised summary of some earlier work on the effect of sex hormones(8) and Vit. D(3) so that the data may be compared.

Material and methods. The subjects had been used in our earlier studies on calcium balance(1). All were free of acute or infectious diseases. Their ages were: DE—88 years, OS—70 years, KO—69 years, KI—72 years, and MC—76 years. Details of clinical status and general procedure of calcium balance determinations have been given(1). Details of diets, adjustment of intake and collection of specimens were the same as in our earlier work on nitrogen balance(9). The diet was similar to Diet No. 3 of this reference. Duplicate portions, each equivalent to all food offered for that day, were analyzed separately each day. Urine specimens, stool specimens and food left by each patient were pooled separately for 5-day periods for analysis. Calcium determinations were made with the Beckman Flame photometer using the procedure previously outlined(3). Calcium balance was determined for 6 to 8 weeks prior to administration of each hormone, and average calculated for this period. The hormone was then administered and calcium balance again determined. After administration of hormone was discontinued, an adjustment period, usually 2 weeks, was allowed before the base period for the next hormone administration was started. Dosages of hormones were: thyroid, 30 mg/day for 60 days; protamine zinc insulin, 10 units/day for 30 days; cortisone, 100 mg/day for 40 days; and ACTH, 30 mg/day for 30 days.

TABLE I. Changes in Calcium Balance with Hormone Administration. All values expressed as mg Ca/kg body wt/day.

Patient	Before hormone admin.				During hormone admin.			
	Intake	Urine	Stool	Bal.	Intake	Urine	Stool	Bal.
Thyroid admin., 30 mg/day for 60 days								
DE	15.3	1.3	16.9	−2.9	15.3	1.2	16.1	−2.0
KO	18.1	3.1	15.7	− .7	18.1	1.8	16.6	− .3
MC	15.6	1.4	18.1	−3.9	15.5	1.5	15.9	−1.9
KI	14.4	1.5	15.9	−3.0	15.5	1.6	15.0	−1.1
OS	14.6	1.9	13.7	−1.0	15.1	1.9	13.3	− .1
Avg	15.8	1.8	16.1	−2.3	15.9	1.6	15.4	−1.1
Insulin admin., 10 units/day for 30 days								
DE	15.3	1.3	16.7	−2.7	14.8	1.4	15.3	−1.9
KO	18.8	1.6	16.4	+ .8	18.4	1.9	15.5	+1.0
MC	15.8	1.4	15.9	−1.5	16.0	1.4	16.6	−2.0
KI	15.1	1.5	14.7	−1.1	14.6	1.5	12.9	+ .2
OS	14.9	1.6	13.5	− .2	15.1	1.6	14.3	− .8
Avg	16.0	1.5	15.4	− .9	15.8	1.6	14.9	− .7
Cortisone admin., 100 mg/day for 40 days								
DE	15.5	2.0	15.8	−2.3	15.9	2.7	15.5	−2.3
KO	17.9	4.0	15.3	−1.4	18.2	4.6	12.6	+1.0
MC	15.6	2.2	16.9	−3.5	15.2	2.6	15.1	−2.5
KI	14.9	1.9	17.0	−4.0	14.0	2.5	14.5	−3.0
OS	12.8	1.6	13.0	−1.8	13.5	2.2	12.3	−1.0
Avg	15.3	2.3	15.6	−2.6	15.4	2.9	14.1	−1.6
ACTH admin., 30 mg/day for 30 days								
DE	14.8	2.1	14.1	−1.4	14.5	2.2	14.7	−2.4
KO	18.1	3.2	15.6	− .7	18.3	3.0	16.1	− .8
MC	15.5	2.3	16.6	−3.4	15.0	2.0	15.3	−2.3
KI	16.1	1.9	16.5	−2.3	15.5	1.8	15.6	−1.9
OS	12.8	1.6	13.0	−1.8	13.3	1.5	13.3	−1.5
Avg	15.5	2.2	15.2	−1.9	15.3	2.1	15.0	−1.8
Vit. D admin., 600 units/day for 40 days								
"	15.4	1.6	15.3	−1.5	15.2	1.5	13.6	+ .1
Estrogen admin. for 60 days								
"	16.5	2.5	14.1	− .1	16.5	2.0	13.7	+ .8
Androgen admin. for 30 days								
"	16.4	3.0	13.7	− .3	16.1	1.7	12.3	+2.1
Estrogen + androgen admin. for 30 days								
"	16.2	2.7	13.7	− .2	16.4	2.0	12.3	+2.1

Results are presented in the Table. All values are expressed as mg of calcium/kg body weight/day averaged over the entire balance period. For comparison, we present the summary of our earlier work on administration of Vit. D (600 units/day for 60 days) on the same 5 subjects, and of our other work on the effect of sex hormones. Details have been presented(8) but the dosages used were as follows: estrogen—1 mg estradiol benzoate in oil, I.M. twice weekly for 60 days; androgen—30 mg testosterone/day for 30 days;

estrogen plus androgen—20 mg testosterone/day plus 1 mg estradiol benzoate twice weekly for 30 days.

There are no marked changes in calcium retention with any of the hormones used. With thyroid and cortisone administration the average balance became less negative, while there was no change with administration of insulin and ACTH. Even with administration of Vit. D there was no large change in calcium retention. The latter figures show that changes in calcium balance need not be large to be detected by the methods used.

Discussion. As summarized from our earlier studies, at the dosage used, estrogen caused only a slight increase in calcium retention. Only administration of androgen (or androgen plus estrogen, which was probably largely an androgen effect) caused any marked increase in calcium retention. There are some changes in calcium intake in individual subjects during different periods, but these are not large, and average intakes were very close, during periods in which each hormone was studied. There are also some variations in calcium balance for individual subjects during various base periods, which are in some instances nearly as great as changes caused by hormone administration. Accordingly, it cannot be said that any hormone except testosterone will cause a significant increase in calcium retention. However, it can be definitely stated that no hormone used will cause decrease in calcium retention.

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Experimental Production of Nephrotic Syndrome Following Renal Vein Constriction in Rats.* (23890)

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Development of a nephrotic syndrome as a result of renal vein thrombosis has been recognized since 1840. However, recurrent, isolated case reports since have made no lasting impressions until recently, when the clinical syndrome has been ably described and relevant literature reviewed in at least 3 excellent articles(1,2,3). The syndrome does not seem to have been reproduced experimentally by occlusion of renal veins in dogs and rabbits. This may be because many of these experiments were of brief duration and performed under severe conditions(1,4). The present report describes a syndrome of massive proteinuria, hypoproteinemia and hypoalbuminemia, hypercholesterolemia, mild hypertension and ascites elicited in rats by sub-total ligation of one renal vein and subsequent contralateral nephrectomy.

Methods. Male Sprague-Dawley rats were used. Experiments are summarized in Table I. The first set consisted of effect of unilateral complete occlusion of left renal vein. The second consisted of the effect of varying degrees of incomplete occlusion of this vein, at a site between kidney and entry of adrenal vein. Partial occlusion was effected by tying a silk ligature firmly around the vein, enclosing a stylet, subsequently withdrawn. The

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TABLE I. Outline of Experiments.

Series	Group	No. rats	Operation	Result
I	1	10	Complete occlusion left renal vein	Partial or complete necrosis
II		14	Partial occlusion	Moderate atrophy, mild proteinuria (Groups 1, 2)
	1	6	Stylet .45 mm	
	2	4	" .7 "	
	3	4	" .85 "	
III			Partial occlusion, later contralateral nephrectomy	
	1	13	Stylet .45 mm; mean wt 94 g	5 survivors: nephrotic syndrome
	2	28		
	a.	9	Stylet .7 mm; mean wt 89 g	6 survivors: 3 with severe proteinuria
	b.	9	" .7 " " 143 g	In effect, "sham-operations"
	c.	10	" .7 " " 244 g	5 survivors: 2 with severe proteinuria

third set of experiments consisted of incomplete occlusion of left renal vein as above and right nephrectomy, 11 to 21 days later. The observations included estimates of body weight, fluid intake, urine flow, proteinuria, arterial pressure and, at end of experiment in Series III, collection of blood for analysis of serum proteins (total and differential), total cholesterol and blood urea nitrogen. At autopsy, kidney and heart were removed for weighing and histological studies.

Results. Complete occlusion of one renal vein (Series I). This operation caused transient proteinuria (to 450 mg/24 hours) with concurrent massive hematuria which subsided on 3rd day; some rats showed mild fluctuant hypertension. The animals were killed on 15th post-operative day; the left kidney was atrophic (mean ratio left/right kidney weight 0.45). Kidneys which had developed collateral venous circulation showed partial cortical necrosis with signs of tubular regeneration; however, medullae were diffusely necrotic and kidneys not functional. There was no mortality. No blood chemical analyses were done.

Partial occlusion of one renal vein (Series II). These experiments were done primarily to discover a degree of partial occlusion which would be compatible with survival after contralateral uninephrectomy. Fourteen rats each weighing 157-200 g were divided into 3 groups in which stylets respectively of .45, .7 and .85 mm diameter were used (Table I). The procedure provoked transient mild proteinuria in most animals of the 2 groups with greater degrees of occlusion; some had persistent proteinuria to 150 mg daily to the time of sacrifice on 15th day. Means of arterial pressures were not significantly increased, although individuals among the first 2 groups showed transient hypertension to 175 mm Hg. The ratios of left/right kidney weight were respectively 0.43, 0.7 and 1.04 in the 3 groups. Correspondingly, kidneys of the 0.85 mm group were histologically normal, whereas kidneys of groups with more intense venous occlusion showed varying degrees of focal necrosis, tubular atrophy and thickening of basement membranes.

Partial occlusion of one renal vein with subsequent contralateral nephrectomy (Series III). Results of Series II indicated that stylets of 0.45 and 0.7 mm were presumably optimal. Rats in Group 1 of Series III weighed 60-104 g. Functional changes after venous occlusion (0.45 mm) corresponded to those described in above paragraph. Eleven days later contralateral nephrectomy was done; 8 rats became oliguric and died during the first post-operative week, showing intense renal atrophy. The 5 survivors showed no immediate symptoms; urine flow and proteinuria persisted at preoperative levels for 4 days; then there began a rise in proteinuria to maxima ranging from 200 to 300 mg daily at 8th to 10th day. The experiment was terminated on 13th day after nephrectomy (Fig. 1). Arterial pressure rose immediately after nephrectomy and hypertension persisted at

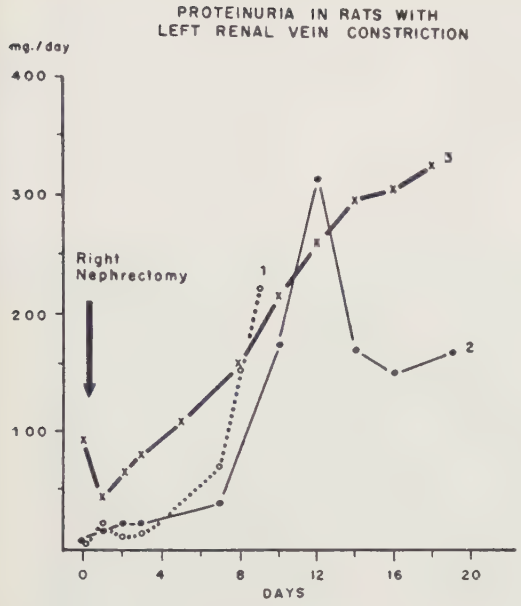


FIG. 1. Proteinuria in Series III. Curve 1, group 1; curve 2, group 2a; curve 3, group 2c.

levels of 188 and 195 mm Hg in 2 rats, while in 3 the hypertension remitted. Serum urea nitrogen and cholesterol increased, while serum protein concentration decreased (Table II). Electrophoretic analyses of serum proteins demonstrated hypoalbuminemia, with increases in alpha and beta globulins but no definite changes in gamma globulin. At autopsy 3 rats had gross ascites, but no peripheral edema. Kidneys of all were large, pale and adherent to abdominal wall, with formation of venous collaterals through the capsule and from the segment of vein lying between ligature and kidney. Increased average heart weight reflected the pre-existing hypertension.

Group 2 consisted of 28 rats divided into 3 sub-groups of different body weights. In *sub-group 2a* contralateral nephrectomy was performed 21 days after partial venous occlusion, at which time urine flow, proteinuria and blood pressure were substantially normal. Three rats died of uremia during the following week; among survivors, 3 showed rapid increases of urinary protein at about 7th day, to maxima of about 300 mg daily at about 12th day; proteinuria persisted at lower levels to end of experiment on 23rd day after nephrectomy (Fig. 1). Blood pressure was not significantly affected. Serum urea nitrogen and protein concentrations were normal, but cholesterol was increased (Table II). At autopsy there was no ascites; the kidneys, although not enlarged, were pale.

In sub-group 2b nephrectomy was performed on 12th day after venous occlusion. Animals were observed for 20 days afterwards.

TABLE II. Chemical and Morphological Changes after Renal Vein Occlusion (Series III).

Group	No. rats	Serum proteins: Total, g/100 ml	Differential in %: Albumin	α-globulin	β-globulin	γ-globulin
1	4	3.01 (2.00–3.65)	29.1 (26.3–32.6)	24.1 (23.8–24.4)	27.4 (23.2–31.2)	18.8 (15.7–24.8)
2a.	3	6.30 (5.59–6.97)	49.9 (43.6–55.8)	16.8 (14.4–19.9)	18.2 (15.4–20.5)	15.1 (14.4–16.0)
b.	7	6.52 (6.25–7.00)	42.4 (39.2–47.3)	16.8 (13.2–21.9)	24.1 (18.8–29.6)	16.7 (12.5–20.2)
c.	2	6.58 (6.40–6.75)	50.3 (49.7–50.8)	12.8 (11.6–13.9)	19.0 (16.6–21.3)	18.1 (17.4–18.7)

Group	No. rats	Cholesterol, mg/100 ml	BUN, mg/100 ml	Kidney, % of body wt	Heart, % of body wt
1	4	91 (81–113)	106 (39–208)	.84 (.69–1.00)	.34 (.28–.40)
2a.	3	100 (83–119)	35 (34– 38)	.62 (.57– .71)	.27 (.26–.29)
b.	7	67 (61– 71)	33 (29– 37)	.64 (.55– .73)	.31 (.22–.36)
c.	2	136 (111–160)	28 (27– 28)	.61 (.60– .61)	.27 (.26–.27)

None showed significant changes. In effect, these represent unintentional "sham-operations."

Sub-group 2c was subjected to right nephrectomy 14 days after partial renal venous occlusion. Five rats died during following week. Of survivors, 3 showed no significant changes. Starting about 3rd day after nephrectomy, 2 developed proteinuria, with maxima of 250 and 660 mg daily at 13th day; it persisted until end of experiment 20 days later. Neither of these showed hypertension, azotemia or significant changes in serum total protein fractions; both showed hypercholes-

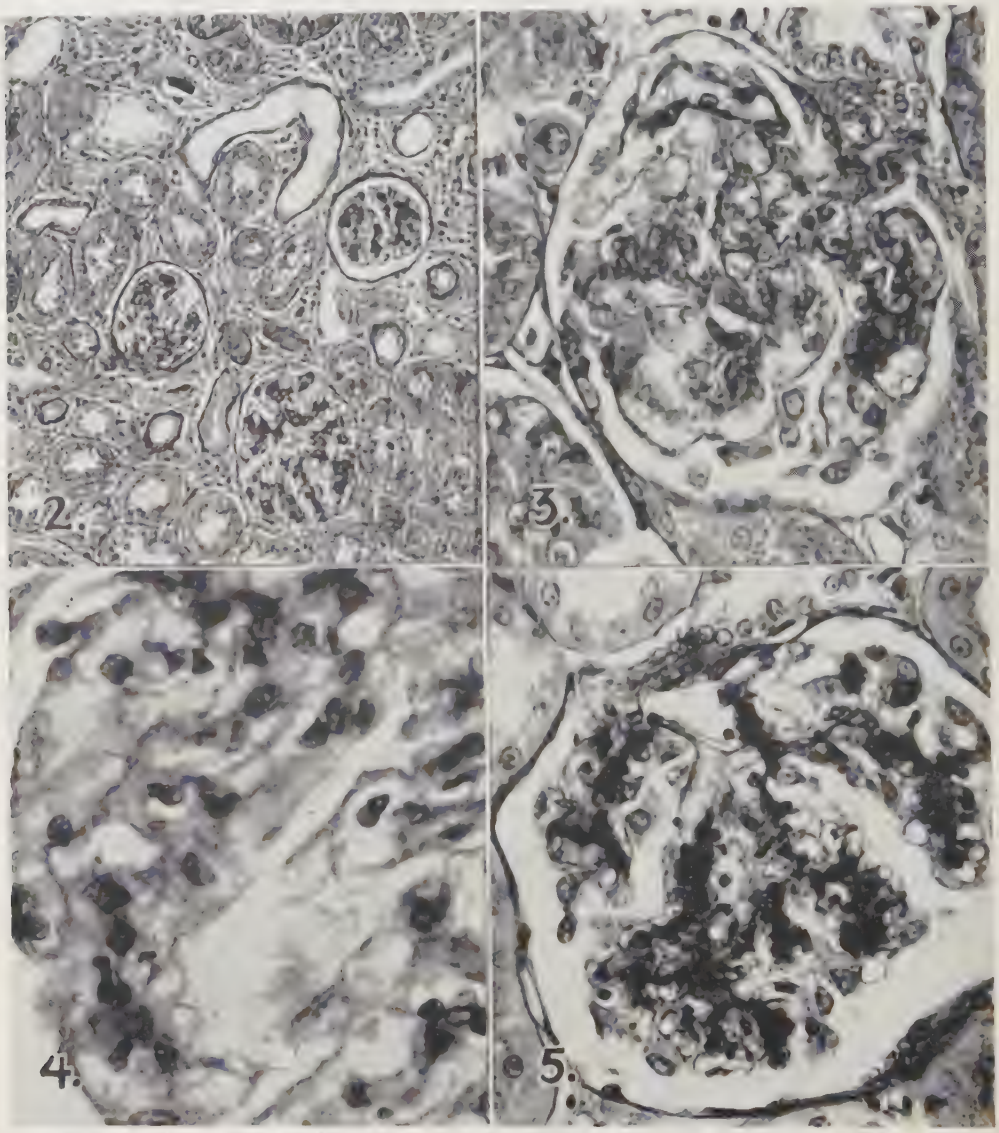


FIG. 2. Kidney showing interstitial fibrosis, tubular atrophy and thickening of basement membranes. PAS, × 160.

FIG. 3. Glomerulus from above kidney showing coalescence of tufts and proliferation of endothelial cells. PAS, × 475.

FIG. 4. Glomerulus showing disruption of normal architecture associated with thickening and fraying of membranes. PAS, × 1000.

FIG. 5. Glomerulus showing marked deposition of PAS-positive material and compactness of tufts. PAS, × 475.

terolemia (111 and 160 mg/100 ml). At autopsy there was no ascites; kidneys were pale.

Histologic findings. The following summarizes observations in the 8 rats which developed severe proteinuria and other aspects of nephrotic syndrome. With one exception, in which there was evident interstitial fibrosis and tubular atrophy (Fig. 2) kidneys appeared normal under low power, showing only a few hyalin or granular tubular casts. Under high power, the only significant changes were found in the glomeruli. These showed condensation and simplification of tuft patterns, coalescence of tufts and fraying and thickening of basement membranes (Fig. 4); glomeruli from animal with interstitial fibrosis showed in addition endothelial proliferation (Fig. 3). Some glomeruli contained deposits of "Schiff-positive" material (Fig. 5). These changes resemble those in clinical cases of nephrotic syndrome, occurring idiopathically or in association with renal vein thrombosis.

Summary. Among 41 albino rats subjected to partial occlusion of left renal vein with subsequent contralateral nephrectomy, 8 developed severe proteinuria and hypercholesterolemia, while 4 had also ascites and hypoalbuminemia. The kidneys were pale, with histological changes limited to glomeruli. These consisted of simplification of tufts, with coalescence, fraying and thickening of basement membranes. This technic requires further standardization, but seems to reproduce the clinical syndrome associated with renal venous thrombosis. To elicite the syndrome, venous occlusion must be incomplete and involve all functioning renal tissue.

ADDENDUM: Since completion of this manuscript, we found that G. Lagrue, B. N. Halpern, P. Milliez and A. Brannelec, *C. R. Soc. Biol.,* 1957, v151, 81, described a similar syndrome, although without noting edema or describing histologic appearance of kidneys, in rabbits subjected to bilateral sub-total occlusion of renal veins.

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Physiologic Chloride Levels in Human Whole Saliva. (23891)

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Chloride concentration of human saliva has been shown to vary in certain systemic disease states(1,2). Dental research interest in salivary chlorides in relation to dental caries has stemmed primarily from the knowledge that chlorides enhance amylase activity and from reports that amylase activity of saliva from caries-active subjects is significantly higher than that from subjects free from carious lesions(3-6). There is diverse opinion as to significant differences in salivary amylase and chloride in subjects with caries activity(7-11) when compared to caries resistant subjects.

Our investigations involve multiple chemical analyses on saliva specimens collected from subjects presenting various oral pathologic states. Since there is considerable disagreement in the literature as to physiologic levels of some constituents under study, we instituted concurrent studies under identical experimental conditions to establish physiologic baselines.

The present study was designed to determine physiologic chloride levels under 2 different forms of stimulation. Also studied were effects of age and sex on chloride concentration and salivary flow rate and the effect of volume on chloride levels.

Procedure. All subjects had recently undergone thorough physical examination and each found to be fit. Times of arising and retiring,

types of food and drink ingested, and environmental conditions were virtually identical in all instances. Oral health status was not a consideration on these otherwise healthy young adults. In the first portion of the experiment (3-RBS) whole saliva samples were collected at 9:00 a.m. from 323 males in 17-19-year age group and from 159 females between 18 and 21 years of age. Salivary flow was elicited with 3 large (size 32) crepe rubber bands, each subject chewing vigorously on the bands for 5 minutes. The advantages of this type eliciting agent have been previously discussed(12,13). In the second portion of the study (NES) 5 minute saliva samples were collected at 9:00 a.m. from 503 males between 17 and 24 years of age and from 179 females in the 18-26-year age group. No exogenous stimulation was employed with these participants. To preclude very small sample size, these subjects were instructed to produce by oral movements, as much saliva as possible during the collection interval. Analyses for variance included all subjects in the 3-RBS portion and 502 males and 164 females in the NES study. The inclusion of only these participants in the latter study was due to the extremely small number of subjects in certain age groups. Chloride concentration was determined by modification of the method of Sendroy(14). Salivary proteins were first precipitated with phosphoric-tungstic acid and the mixture then shaken with excess of solid silver iodate and centrifuged. After addition of sodium iodide to a portion of the supernate, the resultant free iodine was titrated with sodium thiosulfate.

Results. Three rubber band study. The mean volume for 323 male participants was 8.70 ml (S.D., 3.22), while for females was 6.24 ml (S.D., 2.58). No significant differences associated with age were found for either sex, but the difference between volume means for males and females was significant ($P<.01$).

The chloride mean for males was 31.78 meq/l (S.D., 6.96) and that for females was 27.90 meq/l (S.D., 6.22). Confidence limits for chlorides were estimated to be: males—17.9 to 45.7 meq/l; females—15.5 to 40.3 meq/l. No significant differences in chloride levels attributable to age were found for males or for females. However, a significant difference ($P<.01$) was evident when the sexes were compared.

TABLE I. Distribution of Volume and Chloride Means.

Exogenous stimulant	No. subjects	Sex	Volume		Chloride	
			Mean	S.D.	Mean	S.D.
None	503	♂	6.32	2.21	23.28	4.10
3 rubber bands	323	♂	8.70	3.22	31.78	6.96
None	179	♀	4.67	1.30	23.67	3.97
3 rubber bands	159	♀	6.24	2.58	27.90	6.22

Correlation coefficients between volume and chlorides were: males—$r = .20$ ($P<.01$); females—$r = -.15$ ($P<.05$). Thus, for males, volume and chlorides were positively correlated and, for females, these variables were negatively correlated.

No exogenous stimulation study. The mean volume for 503 males was 6.32 ml (S.D., 2.21) and that for females 4.67 ml (S.D., 1.30). No significant volume differences attributable to age were found for males or females. The volume means for males and females, however, were significantly different ($P<.01$).

The chloride mean for males was 23.28 meq/l (S.D., 4.11) while that for females was 23.67 meq/l (S.D., 3.97). Confidence limits were estimated to be: males—15.1 to 31.5 meq/l; females 15.7 to 31.6 meq/l. For males, significant differences ($P<.05$) were found in chloride levels for the 6 age groups tested in the analysis of variance. For females, no significant differences were found in chloride levels for the age groups tested. No significant difference was found in chloride concentrations when the sexes were compared.

The correlation coefficients between volume and chlorides were: males—$r = -.23$ ($P<.01$); females—$r = -.16$ ($P<.05$). Thus, volume and chlorides were negatively correlated for both males and females.

Discussion. Chloride means. White, *et al.* (1) analyzed paraffin stimulated saliva samples from 73 control subjects and reported a mean value of 29.00 meq/l. Chauncey(15), Hubbell(9), and Brown and Klotz(16) also studied stimulated saliva and reported means

of 20, 12.06, and 14.37 meq/l, respectively. Anders(17) studied "resting" saliva in 431 children and found a mean chloride value of 16.25 meq/l. Nonstimulated saliva has also been analyzed by Becks(18), Vladesco(19), and Roskin(11), who reported mean values of 21.04, 33.75, and 17.83 meq/l, respectively. The Russian investigator Popova-Milashevskaia(20) reported salivary chloride mean of 21.88 meq/l in 40 subjects, mode of stimulation not stated.

Sex effects. The significant difference between volume means for males and for females throughout our study has also been noted in earlier work(12,13). In the 3-rbs portion of the present study the female flow rate mean was 71.7% of the mean for males while, in an earlier study(13), in which an identical stimulation procedure was utilized, the female flow rate mean was only 53.4% of that for males. It was postulated that the relatively low flow rate for females might well be due in part to psychologic factors since female subjects did seem much more hesitant to expectorate in view of other participants than did the males. The only procedural variation between the 2 studies was that, in the present study, each group of subjects was given a thorough briefing as to the purpose of saliva sampling and the female subjects were sampled in situations of semi-privacy. The rate of flow for males increased by 0.65 ml/5 min. and that for females increased by 1.94 ml/5 min. under conditions of the present study.

That there was no significant difference in chloride levels attributable to sex in the NES study is in agreement with other reports(17, 20). The significant difference in chloride levels between the sexes in the 3-rbs portion can be explained only when the relationship between volume and chlorides is thoroughly understood.

Age effects. Within the narrow confines of age ranges, age was not found to exert a significant effect upon rate of salivary flow. No significant differences in chloride levels attributable to age were found except for males in the NES study. In this instance the means indicated neither a systematic increase nor decrease with age and are thus felt to be due to chance, which may occur in sampling. Anders(17) concluded that age exerted no influence on chloride levels. Becks(18) found that, under physiologic conditions, excretion of cations and anions in the saliva diminished (except for potassium) between 20-40 years of age but noticeably increased with advance in age. Dancis, *et al.*(21) found that salivary chloride was high at birth, that it dropped rapidly during the first week of life, continued to fall at a much slower rate during the first 2 years, and appeared to stabilize through 10 years of age.

Volume effects. McGrady and Bessman(2) found chloride concentrations to vary markedly with method of collection. Anders(17) presented findings on 9 subjects in whom chloride levels in "resting" and paraffin stimulated saliva were compared. In 5 subjects the chloride concentration was higher in nonstimulated saliva, while the opposite held true in the remaining 4 participants. Brown *et al.*(22) found that the chloride of saliva increased with rate of flow. Thaysen, *et al.* (23), reporting on analyses of parotid saliva from 47 subjects, found that chloride values increased with increases in secretory rate. White, *et al.*(1) could find no highly significant effect of rate of flow on chloride concentrations.

When the 2 portions of the present experiment are compared, volume and chloride levels for both males and females are significantly higher when exogenous stimulation is used. Correlation coefficients for volume and chlorides within each study, however, do not point out a positive conclusion. For females, a negative correlation was noted in both studies, there being no significant difference between correlation coefficients for females for the 2 experiments. For males, however, a highly significant difference between the 2 coefficients was noted. When no exogenous stimulation was used, volume and chlorides were negatively correlated but when 3 rubber band stimulation was employed, volume and chlorides were positively correlated. The indication is that, under conditions of the present experiment, if one attempted to predict volume from chloride levels or chloride levels from volume findings, for males or for fe-

males, the degree of success in prediction would be quite small.

Summary. When whole saliva samples were collected at 9:00 a.m. from 323 males and 159 females under stimulation of 3 large rubber bands and from 503 males and 179 females utilizing no exogenous stimulants, the following conclusions could be drawn: 1. No significant differences in volume attributable to age were noted. Rate of flow for males was significantly higher than that for females. Rate of flow for both males and females was significantly higher when exogenous stimulation was utilized. 2. No significant increase or decrease in chloride values attributable to age was noted. 3. For females, volume and chloride were negatively correlated while, for males, a positive correlation was found with exogenous stimulation and a negative correlation when no such stimulation was used. For both males and females, chloride levels were significantly higher when exogenous stimulants were used.

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Synergistic Effect of Somatotropin with α-Corticotropin on the Adrenal of Hypophysectomized Rats.* (23892)

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Although an antagonism is often operative between adrenocorticotropic hormone (ACTH) and growth hormone (somatotropin) with respect to certain anabolic effects of the latter(1), it is becoming apparent that the 2 hormones may also act in synergism. Cater and Stack-Dunne(2) have studied the combined effect of somatotropin and ACTH in the hypophysectomized rat and demonstrated that the resulting increase in adrenal weight is greater than the sum of increases obtained with each alone. More re-

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cently, we have demonstrated a striking synergism between partially-purified ACTH and somatotropin, reflected in increases in weight of adrenal glands, and have described the lipid changes that accompany administration of these 2 hormone preparations(3). These studies have now been repeated with α-corticotropin (α-ACTH), and the combined effects of somatotropin and this pure peptide are reported here.

Methods. Male rats of the Sprague-Dawley strain were hypophysectomized at 50 days of age; daily injections were begun on fifth post-operative day and continued for 4 days. The somatotropin solution was injected intraperitoneally; thyroxin and α-ACTH were injected subcutaneously, the latter as suspension in a delaying vehicle that contained 5% beeswax and 95% peanut oil. At autopsy, the adrenal glands were removed and immediately fixed in 10% neutral formalin; subsequently, they were dissected free from surrounding fatty tissue and weighed on Roller-Smith torsion balance. For lipid studies, frozen sections of the adrenal, 10 μ thick, were prepared and stained with either sudan black B or with oil red-O. In all instances, sections in the region of adrenal vein were selected for study. The growth hormone was prepared from bovine pituitaries according to procedure previously described(4). It was estimated to contain 0.04 i.u. of thyroid-stimulating hormone (TSH)/mg,‡ but was free from any detectable follicle-stimulating hormone, interstitial cell stimulating hormone or ACTH when assayed at dose level of 10 mg in hypophysectomized rats. The α-ACTH was isolated from sheep pituitary glands(5), and the DL-thyroxin was a product of E. R. Squibb Co.

Results. The adrenal gland of the rat atrophies markedly following removal of the anterior pituitary; nine days after hypophysectomy, changes in weight of gland (Table I) as well as its histology had occurred. In frozen sections, it was evident that the *zonae fasciculata* and *reticularis* were shrunken, and cortical cells of these zones displayed signs of atrophy characteristic of the hypophysectomized animal; a distinct sudanophobe zone was also present. Although the cells of the *zona glomerulosa* retained their normal size, the lipid distribution within them was clearly abnormal. Coarse droplets of sudan-staining material were distributed intermittently in the glomerular cells adjacent to the sudanophobe zone, whereas the cells lying just beneath the outer capsule were completely devoid of lipid.

Treatment with α-ACTH. As expected, α-ACTH promoted substantial weight increases in the adrenal gland of hypophysectomized Sprague-Dawley rats. Administration of a daily dose of 0.025 mg promoted weight increase of approximately 100% over the average adrenal weight of the control group and restored the weight of glands to that of intact control. This dose caused marked hypertrophy of fascicular cells, a considerable diminution in their lipid content, and a redistribution of lipid throughout the *zona glomerulosa*. With a dose of 0.10 mg, hypertrophy of the fascicular cells was accentuated and lipid depletion was nearly complete. Whatever lipid could be detected was present in the *zona reticularis* and the *zona glomerulosa*, although even in the latter it was somewhat depleted. Sinusoidal distention accompanied cellular hypertrophy and no sudanophobe zone was apparent in adrenals of the injected animals. These histological changes were similar to those obtained in hypophysectomized rats of the Long-Evans strain(6).

Treatment with α-ACTH and somatotropin. Somatotropin administered at daily dose levels of 0.050 mg and 0.250 mg increased the weights of adrenal glands from the control value of 16.3 mg to 21.3 mg and 20.3 mg, respectively, the 2 dose levels being equally effective in increasing size of the gland. Despite these changes in weight, no changes in lipid distribution were observed. Furthermore, the dose of α-ACTH and not that of the somatotropin determined the degree of synergism between the 2 hormones. From the Table it is clear that somatotropin given in conjunction with 0.10 mg of α-ACTH was considerably more effective in enhancing adrenal weight than when given with 0.025

‡ We wish to thank Dr. R. W. Bates of the Nat. Inst. of Arthritis and Metabolic Diseases for assay of TSH contamination in growth hormone preparation.

TABLE I. The Effect of α-Corticotropin, Growth Hormone, Thyroxin* and Combinations Thereof on Adrenal Weight of Hypophysectomized Sprague-Dawley Rat.†

Treatment	Daily dose, mg	Body wt, g — Pre-hyp'ed	Day of 1st inj.	At autopsy	Adrenal, mg	Thymus, mg
Normal controls	0	139	151	160	28.7 ± 1.3‡	436 ± 34
Saline	0	145	129	131	16.3 ± .5	338 ± 35
α-corticotropin	.025	139	129	125	28.5 ± .5	0
	.100	143	129	118	43.8 ± 2.2	0
Growth hormone	.050	139	131	141	21.3 ± .9	357 ± 45
	.250	145	131	136	20.3 ± 1.7	374 ± 38
Thyroxin	.005	146	132	132	16.7 ± .5	308 ± 35
α-corticotropin + growth hormone	.025 + .050	149	128	130	32.4 ± 3.0	98 ± 39
Idem	.025 + .250	151	131	134	33.5 ± 2.9	138 ± 36
"	.100 + .050	152	133	134	53.7 ± 2.5	0
"	.100 + .250	136	129	129	54.9 ± 5.3	0
α-corticotropin + thyroxin	.025 + .005	146	132	124	27.6 ± 1.2	133 ± 38

* DL-thyroxin, E. R. Squibb & Co.

† Male rats hypophysectomized at 50 days of age, inj. subcut. at 54 days once daily for 4 days, 4 rats/group.

‡ Mean ± stand. error.

mg of α-ACTH. Histological examination of the adrenal glands from animals treated with given dose of α-ACTH or with the same dose of α-ACTH with somatotropin did not reveal any clear-cut difference in general distribution of lipid or in amount that could be detected within the cortical cells.

Treatment with α-ACTH and thyroxin. As already noted, the somatotropin preparation used in this study did exhibit a limited degree of TSH activity. To establish that the effects observed with somatotropin would not be duplicated by small amounts of thyroxin, one group of animals was injected with 0.005 mg of DL-thyroxin and another group was injected with 0.005 mg of DL-thyroxin and 0.025 mg of α-ACTH. This treatment effected no change in adrenal weight or in lipid distribution.

Discussion. The changes that occur in the adrenal cortex of the rat following hypophysectomy have already been thoroughly described(7), the atrophy being more severe the longer the post-operative interval. Comparisons between results obtained with Sprague-Dawley and the Long-Evans strains reveal that the two are comparable with respect to both adrenal atrophy(8) and to sensitivity to exogenous α-ACTH(6).

An alteration in lipid distribution in the *zonae glomerulosa, fasciculata* and *reticularis* was observed following hypophysectomy. Furthermore, administration of α-ACTH caused redistribution of sudanophil material in the cortical cells of all 3 zones of the adrenal cortex. These observations confirm those reported by Wexler, *et al.*(9) and suggest that the *zona glomerulosa* may not be independent of the control of the anterior pituitary. On the other hand, Deane and Greep and their collaborators have demonstrated that either stimulation or atrophy of the *zona glomerulosa* can be induced with appropriate alterations in the sodium-potassium ratio(10-12); similar effects have been reported for the rhesus monkey(13). Nonetheless, observations of other workers that changes in lipid distribution do occur in the *zona glomerulosa* following administration of ACTH to rat(9,14) and mouse(15,16) suggest some pituitary control. Recently, Lever, in a study of the fine structure of cortical cells with the electron microscope, has observed that although atrophy of glomerular cells after hypophysectomy is

much slower than that of other zones, these cells eventually do show a loss of cytological detail, and that ACTH restores them toward a normal condition(17). This indicates again that the *zona glomerulosa* is influenced by the pituitary and suggests that ACTH is the pituitary factor that is responsible for these changes.

Evidence is also available that secretion by the adrenal gland of both gluco- and mineralocorticoids is subject to pituitary influence. Singer and Stack-Dunne demonstrated that output of mineralocorticoid from adrenal cortex falls markedly after hypophysectomy and that maintenance of the hypophysectomized rat with corticotropin, permits the animal to respond to acute stimulation with ACTH in an essentially normal fashion(18). In addition, *in vitro* studies have shown that when ACTH is added to the incubation medium, some aldosterone-like material, along with a considerable amount of corticosterone, is released by the isolated adrenal(19,20). On the basis of our studies and those cited above, it may be concluded that the *zona glomerulosa* in both rat and mouse is not independent of pituitary control and that the pituitary principle ACTH does act on this zone.

Marked hypertrophy of the adrenal gland can be attained with α-ACTH alone. However, we have observed consistently that the combination of ACTH and somatotropin is more effective in increasing the weight of adrenal gland than is ACTH alone(3). Although there is no suggestion from these results that the capacity of somatotropin preparations to enhance effectiveness of α-ACTH can be attributed to contamination with any other hormone, dosages of growth hormone were selected which provided a maximal degree of synergism with minimal amount of hormone. Dosages of 0.050 mg and 0.250 mg were equally effective under our conditions, and the synergism observed between α-ACTH and growth hormone was related to dosage of α-ACTH. Cater and Stack-Dunne, in investigations on mitotic activity in the adrenal of the hypophysectomized rat, have likewise concluded that growth hormone preparations have an effect on the adrenal cortex of the hypophysectomized rat not attributable to any contamination by ACTH(2). Enlargement of adrenal gland of intact but not of the hypophysectomized rat has also been reported following administration of somatotropin(21). Thus, in these instances where a synergism between growth hormone and ACTH has been demonstrated, the effect obtained appears conditioned by level of either endogenous or exogenous ACTH. More direct studies measuring liberation of corticoids from the adrenal gland, however, have to date provided no support for this contention(18).

Summary. The adrenal gland of hypophysectomized Sprague-Dawley rat showed substantial atrophy 9 days after operation. Histologically-detectable changes in lipid distribution were observed in the *zonae glomerulosa, fasciculata* and *reticularis* following hypophysectomy, and a redistribution of lipid in all 3 zones was effected with α-ACTH. In addition, a synergism between α-ACTH and pituitary growth hormone (somatotropin) was demonstrated, the magnitude of the effect being conditioned by level of exogenous α-ACTH. These results indicate that somatotropin operates synergistically with ACTH when the 2 hormones are administered in combination to the hypophysectomized rat.

The author wishes to acknowledge the able technical assistance of Charles W. Jordan, Jr. and Peggy Woodward Randolph.

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Electrophoretic Studies on Serum Proteins of Young Germfree, Conventional and Antibiotic Treated Conventional Chickens.*† (23893)

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One of the characteristics of the germfree animal is a low level of antibody-containing globulin fractions. Thorbecke *et al.* found that in the germfree chicken a low level of gamma globulin persisted throughout the first 3 months of life. In the conventional‡ bird, during the same time period, this antibody containing fraction steadily increased(1). Meanwhile, we showed that conventional chickens treated orally from birth with 50 mg procaine penicillin G/kg diet develop certain organ characteristics which are qualitatively similar to those seen in germfree animals(2, 3). In essence, these consist of lower weight and less lymphoid tissue content of such organs which are normally in close association with an abundant microbial flora. Comparable changes have been reported by others(4, 5,6,7). Present evidence points to an influence of the antibiotic on composition and/or metabolism of the intestinal flora as the most likely explanation of this effect on the host. It was therefore considered of interest to know whether oral administration of procaine penicillin G to young growing chickens, which was able to produce such definite "germfree-like" morphological features, would have an influence on the globulin fractions of the serum.

Methods. White Leghorn chickens of mixed sexes were used at 35, 75 and 125 days of age. The germfree birds were hatched into the Reyniers germfree system(8,9). After hatch the experimental animals were maintained in commercial brooders and in germfree units respectively. Percentage composition of diet (L-289F) was as follows: yellow corn 50, soybean meal 23, wheat midlings 9, casein 7, alfalfa 2, fish meal 2, meat scraps 2, minerals 4, vitamins and carriers 1. Throughout the experiment steam-sterilized diets were used (121°C, 25 min). Diet and tap water were offered *ad lib.* The present regimen allowed normal growth of both conventional and germfree animals. Procaine penicillin G was administered at 50 mg/kg diet. For further details, see Gordon *et al.*(3). All animal material indicated as germfree tested so up to moment of sacrifice, with the exception of the 125-day-old group. These animals became contaminated accidentally with a pure culture of *Micrococcus sp.*, 1 to 6 days before termination of experiment. Specific name of the contaminant was not determined. As previous experience has shown that in all probability no major changes in serum pattern can be ex-

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‡ Indicating animals of normal stock.

TABLE I. Serum Protein Fractions in Germfree (GF), Conventional (Conv.) and Penicillin-Treated Conventional (PP) Chickens (Sexes Mixed). Under heading Albumin, number of experimental animals is indicated. S.D.M. values are given of data expressed in mg %. The data were obtained with Antweiler's microelectrophoresis apparatus.

Age, days	Units	Albumin			α 1 globulin			α 2 + β globulin			γ globulin		
		GF	PP	Conv.	GF	PP	Conv.	GF	PP	Conv.	GF	PP	Conv.
35	%	78(8)	73(13)	73(9)	3	4	3	10	9	10	9	14	14
	mg %	2775	2509	2315	102	129	95	353	312	332	331	475	443
		± 94	± 55	± 89	± 8	± 15	± 24	± 31	± 26	± 21	± 21	± 39	± 23
75	%	77(5)	68(9)	68(11)	5	3	3	9	9	9	9	20	21
	mg %	2673	2712	2623	170	96	108	306	375	341	321	806	800
		± 79	± 84	± 115	± 31	± 14	± 19	± 27	± 16	± 25	± 43	± 85	± 55
125	%	74*(4)	63(11)	57(10)	4*	2	3	12*	12	12	10*	23	28
	mg %	2753*	2448	2450	160*	94	106	442*	461	506	397*	877	1171
		± 141	± 105	± 72	± 50	± 14	± 10	± 32	± 17	± 31	± 20	± 56	± 77

* Chickens became contaminated with a pure culture of *Micrococcus sp.* 1 to 6 days before sacrifice.

TABLE I(a). Selected Analysis of Differences in Table I.

	Albumin	P	α 1 globulin	P	α 2 + β globulin	P	γ globulin	P
Differences between groups	35 d GF *vs* Conv.	<.01	75 d GF *vs* Conv.	.23	120 d GF *vs* Conv.	.33	35 d GF *vs* Conv.	<.01
	GF *vs* PP	.03	GF *vs* PP	.01			GF *vs* PP	.01
	PP *vs* Conv.	.05	120 d GF *vs* Conv.	.50			75 d GF *vs* Conv.	<.01
			GF *vs* PP	.13			GF *vs* PP	"
	120 d GF *vs* Conv.	.05					125 d GF *vs* Conv.	<.01
							GF *vs* PP	"
							PP *vs* Conv.	.03
Difference with age					GF 35 d *vs* 125 d	.15	GF 35 d *vs* 125 d	.15
					PP 35 d *vs* 125 d	<.01	PP 35 d *vs* 75 d	<.01
					Conv. 35 d *vs* 125 d	"	75 d *vs* 125 d	.40
							Conv. 35 d *vs* 75 d	<.01
							75 d *vs* 125 d	"

pected within the first week after contamination, we have included these data in our results. Serum analysis was performed with Antweiler Microelectrophoresis apparatus using 0.2 ml samples. Total serum protein was determined using the Sargent Kagan Proteinometer.

Results are given in Tables I and I(a) while typical pictures of germfree and conventional chicken serum are given in Fig. 1. Fractions correspond to those given by Sanders *et al.* (10), and Deutsch *et al.*(11). It was, however, possible in practically all instances to establish the separation between fractions alpha 1 and alpha 2, while a clear-cut separation between alpha 2 and beta globulins was not always feasible, especially in older age groups. To avoid arbitrary values, alpha 2 and beta fractions are evaluated together, while alpha 1 globulin is given as such.

Results obtained with Antweiler Microelectrophoresis apparatus differ slightly from those obtained with the classical Tiselius technic. Bierring and Nielsen report that in human serum albumin tends to be about 10% higher, while beta globulin values are lower (12). These observations have been confirmed by us.

Gamma globulin values in germfree birds stayed low during the experiment, confirming earlier results(1). The conventional chickens already had a higher than germfree level at 35 days and showed normal increase with age. Penicillin-fed birds paralleled the untreated conventionals up to 75 days, but thereafter further increase in gamma globulin level seemed to slow down.

In many instances, a separation of gamma

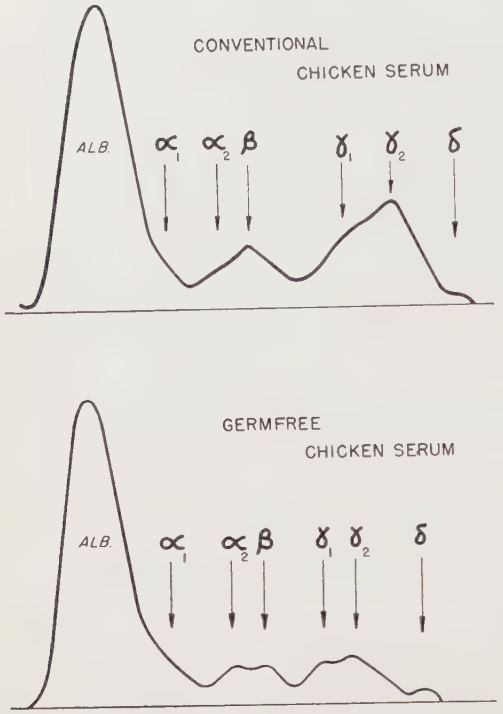


FIG. 1. Electrophoretic patterns of the serum of 75-day-old germfree and conventional chickens.

globulin fraction into gamma 1 and gamma 2 was possible. In all 3 experimental categories, the average amount of gamma 1 then comprised between ¼ and ⅓ of total gamma globulin fraction. No influence of age of animal on this partition was seen.

The alpha 2 + beta globulin fraction increased slightly with age and was apparently more or less alike in all 3 experimental groups. In the alpha 1 fraction, no obvious trends were seen. In the albumin fraction, however, germfree values tended to be higher than conventional and penicillin-treated, both in the 35-day and in the 125-day group.

For evaluation of these results, data on males and females were combined. This was done mainly because in the older age groups not enough germfree material was available to evaluate sexes separately. Analysis of data in the untreated conventional and penicillin-treated groups did not show any obvious influence of sex. This seems to agree in general with the observations of Vanstone *et al.* on chickens of comparable age groups(13). A possible leveling of gamma globulin values in the male after 9 weeks of age, as mentioned by these authors, did not show in the present results.

Discussion. Present data indicate that most or all antibodies which are formed as a reaction to bacterial stimuli are located in the gamma globulin fraction, especially since the increase of antibacterial antibody(14) and of gamma globulin with age go roughly parallel. In the germfree chicken, where no known stimulus of viable bacteria is available, a certain basic level of gamma globulin is still present, though the typical increase with age is virtually absent. The question whether this represents mostly "inert" gamma globulin or the product of an antibody response against unknown antigenic stimuli cannot be answered satisfactorily.

Morphological differences between germfree and conventional animals, such as reduced weight of small intestine and ileocecal lymph nodes, are well established at 35 days of age(3). In that case the neutralizing effect of penicillin on flora stimulation evidently makes itself felt from the start. However, antibody titers in normal conventional chicken are known to build up slowly(14), a fact which apparently is reflected in the gradual build-up of gamma globulin content over the germfree level. Here, the effect of the antibiotic seems to be virtually absent for the first

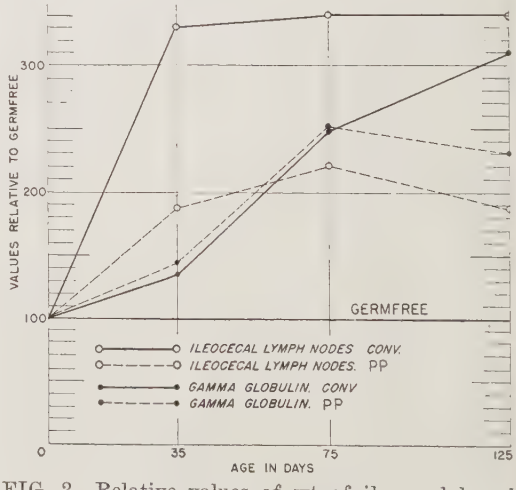


FIG. 2. Relative values of wt of ileocecal lymph nodes and of serum gamma globulin content of conventional (Conv.) and procaine-penicillin treated conventional (PP) chickens, setting germfree values at 100. All original data expressed in mg %.

3 months, while only at the age of 4 months, signs of a neutralizing effect became apparent (Fig. 2).

Evaluation of fractions gamma 1 and gamma 2 globulin was done wherever possible in the hope of finding one fraction containing mostly "germfree" gamma globulin along with another containing mostly flora induced gamma globulin. It was found, however, that gamma 1 and gamma 2 participated to about the same extent in all 3 experimental groups in all age classes investigated. There is a tendency for the germfree animal to have a somewhat higher serum albumin level. Our experiments have shown that this is definitely so in the rat and the same phenomenon manifests itself here in 35-day and 125-day groups. A possible explanation may be to regard the higher albumin content in germfree as compensating for the lower gamma globulin level to maintain the osmotic equilibrium.

As these experiments were mainly designed for a detailed study of morphological and related changes brought about by feeding of penicillin, the emphasis was placed on younger age groups and no data beyond 125 days are available. It is felt, however, that the influence of feeding penicillin on the gamma globulin level of the conventional chicken should be confirmed in animals of older age groups. Work along these lines is in progress.

Summary. The effect of feeding procaine penicillin G (50 mg/kg diet) on the electrophoretic pattern of serum of young conventional chickens was studied and compared with data obtained from germfree animals. While the effect of the antibiotic on morphological characteristics of conventional chicken made itself felt after one month, up to the age of 2½ months no differences were seen in the electrophoretic pattern. Both groups showed an equal increase in gamma globulin over the level found in serum of the germfree animal. At 4 months, however, the antibiotic-treated birds showed lower gamma globulin values than the untreated group, though the level was still substantially higher than found in germfree serum.

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Potentiation Between Cholinesterase Inhibitors.* (23894)

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Simultaneous administration of insecticides EPN (ethyl-p-nitrophenyl thionobenzenephosphonate) and malathion (S-(1,2-dicarbethoxyethyl) 0,0-dimethyl dithionophosphate) to rats and dogs has been reported to result in potentiation of their toxicities(1). This is thought(2,3) to be due to inhibition by EPN of detoxification of malathion by the liver. Since many other organic phosphate anticholinesterases are widely used as insecticides it is important to determine whether other combinations also show potentiation to enable one to evaluate hazards involved in handling these agents and in consuming food from crops sprayed with them. Data are presented here which show that several combinations of anticholinesterase insecticides exhibit potentiated toxicities, and the results of cholinesterase studies are correlated with this phenomenon.

Methods and materials. Young adult male Swiss-Webster mice fasted 16 to 20 hours were used in toxicity experiments. The anticholinesterase agents tested were technical samples of EPN (89%)† malathion (95%),‡ chlorothion (p-nitro-m-chlorophenyl dimethyl thionophosphate)§ BFP (bis-dimethyl-amido fluorophosphate)§ and phostex (mixture of bis-(dialkoxyphosphinothioyl) disulfides).‖ All anticholinesterases were given orally in corn oil and combinations were administered simultaneously except where otherwise noted. Animals were observed at least 48 hrs following injections and times of death recorded. Brain and liver tissue used for cholinesterase estimations were obtained from Wistar rats (200-300 g). In liver incubation studies the general procedure of DuBois, Doull and Coon (4) was used. Liver slices (15 to 30 mg dry wt) were incubated for 1 hr in a Krebs phosphate medium with and without added aqueous solutions of anticholinesterase. One-tenth portions (0.3 ml) of these incubates were then added to flasks containing 2.4 ml of brain homogenate and allowed to incubate 1 hr. Cholinesterase determinations were then performed manometrically by the method of DuBois and Mangun(5) with 0.3 ml 0.1 M acetylcholine chloride as the substrate.

Results. Potentiated toxicity between 2 compounds was assumed present if non-toxic doses of each, when combined, produced a greater mortality than did twice these doses of either compound alone. Those combinations which showed potentiation by this definition are recorded in Table I. EPN, malathion, chlorothion and phostex in all possible paired combinations showed potentiation. EPN plus malathion showed greatest potentiation and EPN plus phostex the least. Potentiation was also seen with these agents in numerous other combinations of dosages not recorded in Table I. It was also observed that when EPN and malathion were given 4 hrs apart the potentiation was almost as great

TABLE I. Combinations of Anticholinesterases Showing Potentiation in Toxicity.

EPN	Malathion	Chlorothion	Phostex	No. of mice	% mortality
mg/kg					
30				10	10
20				10	0
	500			19	21
	250			12	0
		1000		10	70
		500		10	0
			2000	9	11
			1000	11	0
10	125			11	91
10	60			10	30
10		250		10	40
20			1000	7	43
10			1000	10	20
	250	180		10	80
	125		500	8	75
		250	1000	10	60

* This investigation was supported in part by Office of Surgeon General, Department of Army.

† EPN was supplied by E. I. du Pont de Nemours & Co.

‡ Malathion was furnished by Am. Cyanamid Co.

§ Chlorothion and BFP were given to us by Dr. K. P. DuBois, Univ. of Chicago.

‖ Phostex was provided by Niagara Chem. Division, Food Machinery and Chem. Corp.

TABLE II. Inactivation of Malathion, Chlorothion and Phostex by Rat Liver Slices.

Anticholinesterase	Molar conc.	% brain cholinesterase inhibition Incubated without liver slices	with liver slices
Malathion	1×10^{-4}	64	23
	5×10^{-5}	38	0
Chlorothion	1×10^{-4}	57	36
	5×10^{-5}	40	0
Phostex	1×10^{-5}	95	14
	5×10^{-6}	40	0

as when given simultaneously, and even when EPN was administered 24 hrs before malathion this pair still showed some potentiation. This is in accord with the finding that a single dose of EPN inhibits ability of rat liver to detoxify malaoxon (oxygen analog of malathion) for as long as 72 hrs(3). Pairs of anticholinesterases tested which did not show definite potentiation were: malathion combined with OMPA (octamethyl pyrophosphoramide), with parathion (p-nitrophenyl diethyl thionophosphate) or with physostigmine; EPN plus parathion, EPN plus OMPA, and OMPA plus parathion. Malathion plus BFP appeared to be a borderline case.

Since chlorothion and phostex have relatively low toxicities, the possibility was considered that they, like malathion, might be inactivated by the liver. As shown in Table II we confirmed the inactivation of malathion by liver and also found that chlorothion and phostex are partially inactivated. The results recorded are the averages of 2 to 4 determinations. It appears that phostex, the least toxic of all of the anticholinesterases employed, is also the most extensively inactivated by the liver. It was anticipated that EPN would inhibit inactivation of chlorothion and phostex, as it does in the case of malathion(2,3), thereby accounting for potentiation observed with these agents. Since malathion, chlorothion and phostex are all inactivated to some extent by liver a competition for sites of inactivation seems possible when combinations of these 3 inhibitors are administered to mice. This competition could explain the observed potentiation in toxicity. It was first seen, in confirmation of an earlier report(1), that addition of EPN and malathion to brain homogenates without prior incubation with liver slices did not cause a potentiation of cholinesterase inhibition. *In vitro* liver incubation studies were then performed to see whether potentiation of toxicity by various combinations of cholinesterase inhibitors could be correlated with *in vitro* inhibition of inactivation of certain of these agents by liver slices. When anticholinesterases were first incubated with liver slices, either alone or combined, potentiation of brain cholinesterase inhibition was observed with several combinations (Table III). The results are averages of 2 determinations. Each combination that exhibited potentiated inhibition of cholinesterase also showed potentiated toxicity (Table I). None of the combinations tested showed as great a potentiation of enzyme inhibition as did EPN plus malathion, and this pair also produced the most marked potentiation of toxicity (Table I). Preliminary results indicate that OMPA does not inhibit inactivation of malathion by liver slices since this combination showed no potentiated inhibition of brain cholinesterase after incubation with liver slices. This agrees with the observed lack of potentiation in toxicity when OMPA and malathion were combined.

TABLE III. Potentiation of Brain Cholinesterase Inhibition by Combinations of Anticholinesterases Previously Incubated with Liver Slices.

Anticholinesterases	Molar conc.	% cholinesterase inhibition
EPN	1×10^{-5}	0
Malathion	1×10^{-4}	0
EPN + malathion	1×10^{-5} + 1×10^{-4}	78
EPN + malathion	1×10^{-5} + "	48
EPN	1×10^{-6}	0
Chlorothion	5×10^{-5}	17
EPN + chlorothion	1×10^{-6} + 5×10^{-5}	32
EPN	1×10^{-6}	0
Phostex	1×10^{-5}	14
EPN + phostex	1×10^{-6} + 1×10^{-5}	45
Malathion	1×10^{-4}	9
Chlorothion	1×10^{-5}	0
Malathion + chlorothion	1×10^{-4} + 1×10^{-5}	65

Discussion. The data presented demonstrate that combinations of anticholinesterases other than EPN and malathion exert potentiated toxic action in mice. In each combination showing potentiation at least 1 of 2 compounds is inactivated by liver. Inactivation of malathion, chlorothion and phostex by liver is undoubtedly the reason for their low toxicity since they are fairly strong cholinesterase inhibitors when added directly to brain homogenates. This inactivation of chlorothion by liver slices is not in accord with the report(2) that this compound is unaltered by liver homogenates, a discrepancy which may be due to differences in methods used. We observed that EPN inhibits inactivation of chlorothion and phostex by liver as has been reported for malathion(2,3). Combinations of malathion and chlorothion showed potentiation in toxicity and cholinesterase inhibition possibly because they mutually inhibit their inactivations through competition for the same enzyme site. Thus in order for potentiation to occur it appears that one or both compounds must inhibit inactivation of the other.

It is not yet possible to explain why EPN inhibits inactivation of malathion whereas OMPA does not. Purified EPN and OMPA are oxidized by liver from weak cholinesterase inhibitors to potent inhibitors(4,6), and one might expect them to affect inactivation of malathion similarly. Our technical sample of EPN differs from purified EPN in that it possesses some *in vitro* anticholinesterase activity, but after incubation with liver slices it is a more potent inhibitor. OMPA and other anticholinesterases in high concentrations may inhibit inactivation of malathion, chlorothion or phostex as does EPN, but the concentrations required may produce enough cholinesterase inhibition as to be themselves toxic, thereby masking any interference with the inactivation of malathion. This could account for the apparent lack of potentiation between OMPA and malathion.

Evidence indicates that detoxification of malathion involves hydrolysis of ester linkages in the 1,2-dicarbethoxyethyl side chains (2,7). It has been suggested, therefore, that EPN may inhibit detoxification of other drugs, besides malathion, which contain ester groups (3). Chlorothion and phostex do not contain ester groupings but do contain alkoxy groups which suggests that the inactivation of these agents may involve dealkylation. The possibility thus arises that EPN also inhibits dealkylations in the body.

The observations described here suggest the necessity of focusing broader attention upon hazards which may be involved in simultaneous exposure to, or ingestion of, through any source, 2 or more anticholinesterase agents which are used as insecticides. Extensive manufacture and wide dissemination of a great variety of these agents has created the complex and important problem of protecting workers and the food-consuming public against the potential dangers of these materials in their environment.

Summary. Organic phosphate insecticides EPN, malathion, chlorothion and phostex, in all possible paired combinations administered simultaneously to mice showed potentiation in their oral toxicities. Some potentiation was observed even when EPN was given 24 hours before malathion. Malathion plus BFP showed borderline potentiation, whereas malathion combined with OMPA, parathion or physostigmine showed no potentiation, nor did EPN plus parathion, EPN plus OMPA or OMPA plus parathion. Malathion, chlorothion and phostex were partially inactivated by incubation with rat liver slices. EPN inhibited inactivation of malathion, chlorothion and phostex, producing an *in vitro* potentiation of cholinesterase inhibition. Potentiation in all cases found so far appears to be caused by interference by one compound with inactivation of the other by liver, or by a mutual interference with inactivation through a competition for a specific enzyme site.

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Tissue Culture and Circulating Myeloblasts of Avian Leukemia Studied in Electron Micrographs of Ultrathin Sections.* (23895)

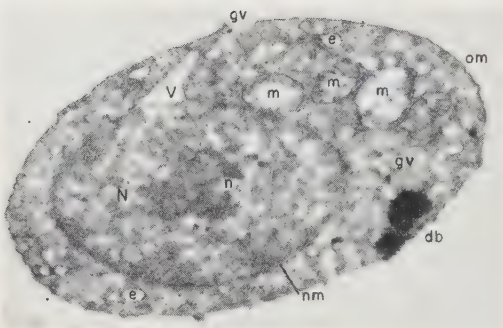
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In blood of chickens with virus-induced myeloblastosis, there occur primitive cells of myeloid origin(1,2). Recent studies(3) have shown that such cells can be maintained for long periods in tissue culture. Under these conditions virus particles characteristic of the disease are liberated in large numbers into the culture fluid, as estimated by direct particle count in electron microscope and by measurement of enzymatic activity of the agent to dephosphorylate adenosine triphosphate. The source of the virus was presumably the primitive cells. In this report there are described the results of studies made by means of technics of ultrathin sectioning† with the object of obtaining information about the cell-virus relationships involved in the process. The principal materials examined were myeloblasts taken directly from the circulation and the cells after various intervals of culture. For purposes of comparison, there were investigated, also, sections of spleen, bone marrow and liver.

Material and methods. The materials were obtained from birds with avian myeloblastosis produced with virus of the B.A.I., a strain studied(4) for several years in this laboratory. Experimental induction of the disease was effected by intravenous injection of 0.1-ml volumes of filtered whole or diluted plasma from previous passage chickens. Myeloblasts were obtained as already described(3) by centrifugation of heparinized blood. Donor birds, in advanced stages of the disease, were selected for high blood content of myeloblasts (about 1 to 2 million/mm^3) as judged from routine blood smears and high plasma virus concentration (about 10^{12} particles/ml) determined by enzyme activity(5). Tissue cultures of the myeloblasts were established and maintained in the usual way(3) in medium consisting of Gey's saline solution containing 5 to 20% chicken serum and added glucose in 0.5% concentration. The cultures were prepared in 5-ml volumes with about 10^7 cells/ml in 50-ml flasks and incubated at 37°C on the gyratory shaker. Fluids were changed at 2 to 4-day intervals. Two kinds of samples of cells were taken for thin sectioning; namely, free floating cells collected by centrifugation of culture fluid; and attached cells scraped from walls of flasks. Samples were taken from 4 different series of cultures at intervals of 0 to 77 days. Each sampling was from 2 or 3 flasks carried in parallel in each series. All were fixed for 3 hours in the cold in 2 ml of 1% osmic acid buffered to pH 7.4. Myeloblasts from centrifuged blood were delivered in drops of about 1 mm diameter from vaselined fine-tipped pipettes directly into the osmic acid solution. Pieces of spleen, bone marrow and liver of about 1 mm^3 were taken from 5 diseased birds in late stage of disease. Studies were made, also, on spleen, liver, bone marrow and white blood cells from 2 untreated birds of comparable age. Procedures for preparation of all specimens for thin sectioning were essentially those of Palade(6). The fixed samples were dehydrated with alcohol, imbedded in methacrylate and sectioned with

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N = nucleus, n = nucleolus, nm = nuclear membrane, pm = plasma membrane, m = mitochondria, e = ergoplasmic reticulum, gv = grey vacuole, db = dense body, om = osmophilic plasma membrane, p = absent plasma membrane, V = vacuole, v = virus, b = inclusion of small virus-like particles, ib = inclusion bodies.

FIG. 1. Thin section of a typical myeloblast from peripheral blood of chicken in late stage of myeloblastosis. Magnification 10,550 ×

the Porter-Blum microtome.

Results. Circulating myeloblasts. Examination has been made of about 2500 thin sections of myeloblasts taken from blood without treatment other than centrifugation at low speed to separate primitive elements from red blood cells. The findings were quite uniform from one donor to another and are illustrated in the micrograph of the myeloblast in Fig. 1. The nucleus, surrounded by a double membrane, contains a prominent nucleolus and a loose chromatin structure showing a tendency to margination. Such cells from diseased birds differed in several respects from myeloblasts in bone marrow of apparently normal chickens. Cells in the former case showed swelling and fragmentation of mitochondria and vacuolization of cytoplasm. Densely staining bodies were of frequent occurrence in the cytoplasm, sometimes attached to the plasma membrane. While in this section the bodies were stained as densely as fat vacuoles, in others, they seemed less osmophilic and contained structures resembling virus identical in morphology with virus particles seen outside the cells. In 66% of the sections from this source bird, there were 2 or 3 of the bodies, and the ratio in sections from other birds was directly related to virus content of the plasma. Vacuoles containing lightly osmophilic material and surrounded by a single wall ("grey vacuoles") are scattered in the cytoplasm. These, on high magnification, contained many small round particles with a dense center. As shown, too, in Fig. 1, a part of the plasma membrane was frequently abnormally osmophilic.

The most remarkable feature of the findings was the *very low incidence of typical virus inside the myeloblasts.* Although virus particles were seen in areas away from cells, in only 4 instances of all sections of these myeloblasts were such particles seen inside the cells. In these cases the virus was situated in vacuoles close to the plasma membrane.

Tissue culture myeloblasts. In preparation for tissue culture, the myeloblasts were washed several times with Gey's salt solution to eliminate plasma virus. The efficacy of the procedure was shown by absence of enzyme activity of samples of fluids taken at 0 culture time. Thin sections of cells taken at 0 time were of essentially the same appearance as that of cells in the circulation as shown in

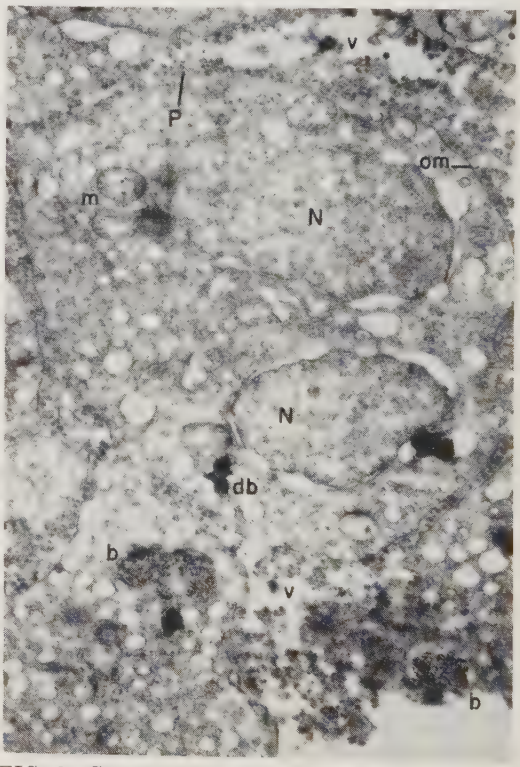


FIG. 2. Cells from a culture of myeloblasts after 77 days. A large amount of virus (v) surrounds the cells. Magnification 13,000 ×.

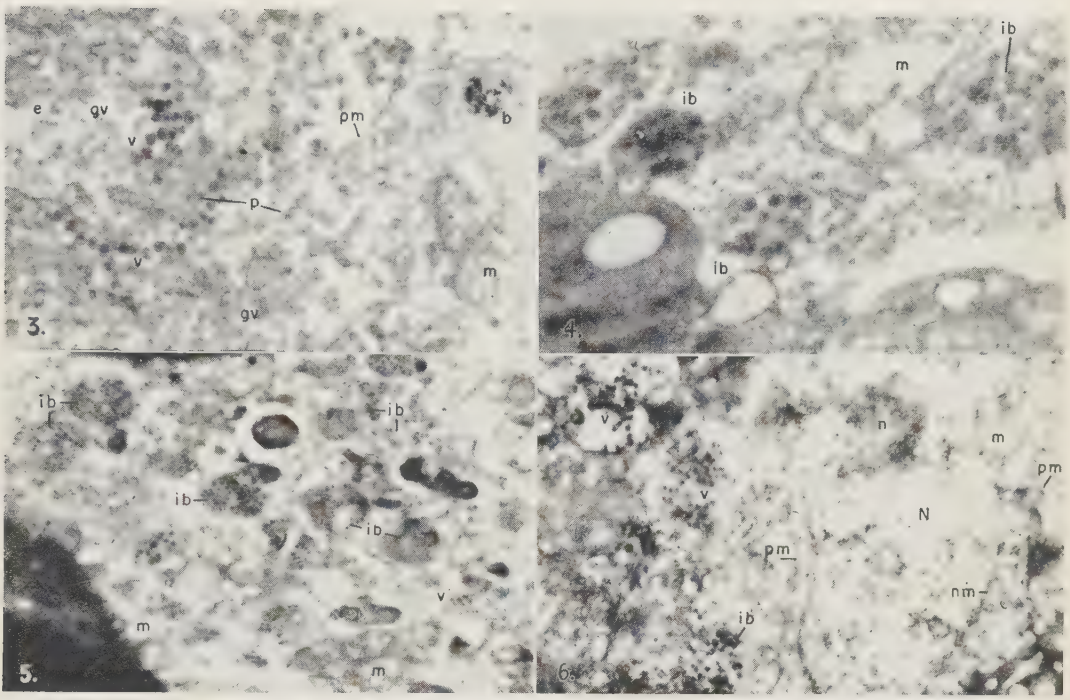


FIG. 3. Enlargement of a portion of a cell from a 58-day tissue culture of myeloblasts of the same series as Fig. 2. Virus (v) has accumulated both around the border of the cell and also inside the cytoplasm. The plasma membrane (pm) is missing in places (p). Magnification 20,000 ×.

FIG. 4. Portion of cytoplasm of an unidentified cell from bone marrow in myeloblastosis. Several inclusion bodies (ib) containing virus are shown. Magnification 30,400 ×.

FIG. 5. Portion of macrophage-like cell from bone marrow in myeloblastosis showing many inclusion bodies (ib) and free virus (v). Magnification 10,000 ×.

FIG. 6. Cells resembling reticular cells in spleen of the same bird as Fig. 5 showing similar inclusion bodies (ib) and free virus (v). Magnification 10,500 ×.

Fig. 1. Vacuolization of the cells seemed greater after washing. Virus of characteristic appearance was rarely seen either inside or outside the cells.

Cell specimens taken at intervals after 2 to 77 days of culture were of similar appearance whether they were free-floating in the culture fluid or had been attached to the flask walls. An example of findings in some sections is shown in Fig. 2 (floating cells after 77 days culture). The outstanding finding here is accumulation of large amounts of virus of characteristic appearance outside but in close association with the surface of the cells. In other sections, much less virus was accumulated about the cells although the concentration in fluid was high as indicated by enzyme activity. The factors determining distribution of particles in the culture are not yet known. Little of the agent, clearly recognizable as such, was within the cytoplasm as illustrated in Fig. 3, which is a higher magnification of a cell (from the flask wall) from the same culture series taken after an interval of 58 days of incubation.

A number of the dense bodies demonstrated in Fig. 1 are present in Fig. 2. Structures similar to the "grey vacuoles" already mentioned but containing more osmophilic small virus-like particles are present (b) in Fig. 2 and also in Fig. 3. The plasma membrane was osmophilic in some areas (om) and missing in others (p). Where the cell membrane was absent, virus particles were seen often apparently imbedded in the cytoplasm near the cell edge. In general the morphologic features of the cultured myeloblasts are recognizable, but variations in the appearance of the cells and in the number of degenerating cells were observed in different specimens. In Fig. 2,

the nucleus of the central cell is bilobed, and the perinuclear space, endoplasmic reticulum and mitochondria are more swollen than in the normal cells of the bone marrow. Some of the cells in other sections contained large amounts of osmophilic material in both the cytoplasm and the nucleus. On higher magnification of these denser areas, entities similar in shape to small virus particles could be made out.

Cells of the bone marrow. In contrast to the findings with the myeloblasts, many cells in the bone marrow contained very large accumulations of virus. An unidentified cell (not a myeloblast) in Fig. 4 contains inclusions of characteristic virus particles. As was usual with this material, much of the virus was of indistinct morphology. Dense bodies and swollen mitochondria were also present in a neighboring cell. In the bone marrow virus could also be found in association with very vacuolated myeloblasts with ruptured plasma membranes. Large cells resembling macrophages or reticular cells were crowded with large numbers of inclusion bodies filled with virus (Fig. 5). Structures resembling virus were not seen in any intact myeloblast in the bone marrow in this series.

Cells of spleen. In Fig. 6 there are inclusions of virus in cells with a large amount of cytoplasm, presumably reticular cells in the spleen of the same bird that provided the bone marrow of Fig. 5. Although a large part of the spleen was occupied by degenerating myeloblasts, no virus was positively identified in them.

Liver. The liver in some birds showed extensive changes. Very large numbers of myeloblasts were observed, and there was some necrosis of liver cells. Inclusion bodies filled with typical virus and virus free in the cytoplasm were present in large degenerate cellular elements which may have been Kupffer cells. These contained much osmophilic material, which under high resolution, appeared to be made up of small virus-like particles. The liver cells contained the same kind of grey vacuoles as those described already in myeloblasts and in a few instances, virus was seen in vacuoles in cells resembling liver cells.

No evidence was seen of virus-like particles in the spleen, bone marrow or liver of uninfected birds of the same age and strain as those diseased with myeloblastosis.

Discussion. The present observations are of significance in 2 principal aspects: (1) the infrequent demonstration of typical virus particles in myeloblasts either those in circulation or those in tissue cultures; and (2) the great difference between this lack of virus in myeloblasts and the amounts of agent found in cells other than myeloblasts in bone marrow, spleen and liver.

That virus is regularly liberated into the fluid of tissue cultures of myeloblasts has been well established(3). In some cultures, the agent shed into the fluid numbered as many as 2000 particles/cell in a 48-hour culture period. The findings provide additional substantiation of the results obtained by enzyme measurement and particle count. Because of this, it has been the more remarkable that practically none of the agent was present in distinctive form within these cells either before or after they were cultured. The studies have not been carried far enough to give a clear indication of the nature of virus precursors in the cells or the site of virus synthesis. There may be mentioned for further consideration the frequently occurring dense bodies, which usually represent fat, and greyish vacuoles in which there could be seen suggestions of ovoid structures or very small particles that might be the very early forms of the agent. Virus was occasionally found in structures with the appearance of vacuoles, but whether the particles had been formed there or simply taken up by the cell could not be determined.

With affected cells other than myeloblasts, the findings were quite different. Virus of distinctive morphology was frequently to be seen in very large amounts inside such cells. More often than not it was present in clearly defined walled structures with the appearance of inclusion bodies, though some appeared scattered in the cytoplasm. The structures observed in the non-myeloblastic cells were like those which have been seen by other investigators in the liver and spleen in lymphomatosis(7) and in sections of tissues from birds diseased with the same strain of myeloblas-

tosis virus(8) employed in the work described here. Virus particles of morphology identical with that of the agent of myeloblastosis studied in the present work have been seen in the spleen and bone marrow of chickens with erythroblastosis(9). In myeloblastosis and erythroblastosis, the agents of which are related though distinct(4,10), the virus particles observed inside the cells have been identified with the infectious entity in isolated and purified preparations(11).

The intracellular particles seen in the present work are of particular interest since the virus of myeloblastosis has been identified in purified preparations by physical, chemical and immunologic procedures(2). Studies of ultrathin sections of such purified virus have revealed(11) an essentially homogenous population of particles of characteristic structure. Particles seen inside the cells exhibited the same morphology as the particles in ultrathin sections of the purified preparations. In addition, the morphology of intracellular particles was identical with that outside the cell in the same micrographs. Because of the identity in morphology(11), of the characteristics of the disease occurring in the donor chicks, and of the biologic and morphologic characteristics of the agent liberated in tissue culture, there would appear to be a sound basis for judging that the intracellular structures are representative of the agent of myeloblastosis.

From these results it may be judged that the myeloblasts not only liberate virus but actively support the proliferation of the agent in tissue culture. It is further indicated that the final conversion of precursor substance (not recognizable in the micrographs) to virus like the extracellular particles occurs rapidly and that the agent quickly leaves the cell. The lack of fully formed virus particles in the cytoplasm suggests that a large part of at least the final process may occur close to or at the plasma membrane. This possibility has already been considered(12) in relation to the Rous sarcoma in which virus particles were present mostly at the surface outside the cell. In some micrographs(12) the particles of Rous sarcoma virus appeared to be imbedded in the most superficial cytoplasm in a manner very similar to that observed with the myeloblasts. In types of cells other than myeloblasts the processes of proliferation and liberation appear to differ from those in myeloblasts in degree if not in kind. Virus was not all liberated immediately from such cells but might be crowded in large amounts in inclusion structures and as individual particles throughout the cytoplasm. In view of this evidence of massive cellular involvement, it is small wonder that the virus may reach concentrations of more than a trillion particles/ml plasma.

The findings with myeloblasts, especially those from blood, are of much practical significance in relation to studies on possible relationships of viruses to malignancy of presently unknown etiology. The myeloblasts are malignant derivatives of virus-infected precursor cells. That they, themselves, are infected and can support virus liberation at high levels was demonstrated by the tissue culture experiments. Yet this state and activity might well have been overlooked in a cursory examination of the circulating cells. Analogous conditions have been encountered not only with the Rous sarcoma(12,13) but with the Murray-Begg endothelioma(14). In the former few particles are to be seen inside the cells, and the studies thus far have shown only extracytoplasmic agents in association with the latter. It is well to recognize probable major differences in the mechanism of proliferation of the various tumor viruses, evidence for which is already considerable. Much virus is seen in the cytoplasm of the cells of the mouse mammary carcinoma(7,15) and in those of mouse lymphatic leukemia(7); the antigen of the rabbit papilloma virus appears to be concentrated in the cell nucleus(16); and the results of the present work demonstrate considerable differences in the reaction of different types of cells to the same virus in the same host. The variations serve well to emphasize the difficulties to be encountered in the search for viruses in association with tumors not known to be caused by filterable agents.

Summary. Studies have been made by electron microscopic examination of ultrathin sections of circulating myeloblasts of avian

myeloblastosis and of these cells in tissue culture. Examinations were made, also, of tissues of spleen, bone marrow and liver of the diseased birds. The results showed that intracellular virus of typical appearance was very rarely seen in myeloblasts of the circulation and only very infrequently in the cells after culture *in vitro*. This was the remarkable feature of the findings, since the cells liberate virus at a high rate for long periods in tissue culture, and large amounts of *extracellular* virus were associated with the cultured cells. In contrast, affected cells not myeloblasts in spleen, bone marrow and liver contained intracytoplasmic virus in high concentrations, most of it in inclusion-like bodies. The significance of the results was discussed in relation to similar findings with the Rous sarcoma and Murray-Begg endothelioma and to exploratory studies for the demonstration of virus in tumors of presently unknown etiology.

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Excretion of 17-Hydroxycorticosteroid Hormones by Pantothenic Acid Deficient Guinea Pigs.* (23896)

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Although recent investigations have clearly established the important relationship of pantothenic acid to adrenal steroid secretion in the rat(1-5), the role of this vitamin in adrenocortical function in other animals has not been studied. The purpose of this report is to describe investigations of secretion of adrenal steroid hormones by guinea pigs made deficient in pantothenic acid. The results demonstrate a diminished excretion of 17-hydroxycorticosteroids in response to ACTH administration.

Materials and methods. Young, male guinea pigs (Hartley Strain) weighing 150 to 250 g were used. Animals were housed individually in metabolism cages, weighed frequently, and 24-hour urine samples collected. Each urine specimen was diluted to 100 ml with distilled water and a 5 ml aliquot used for determination of 17-hydroxycorticosteroid hormone content by the method of Silber and Porter(6). To obtain biochemical evidence of pantothenic acid deficiency, coenzyme A analyses

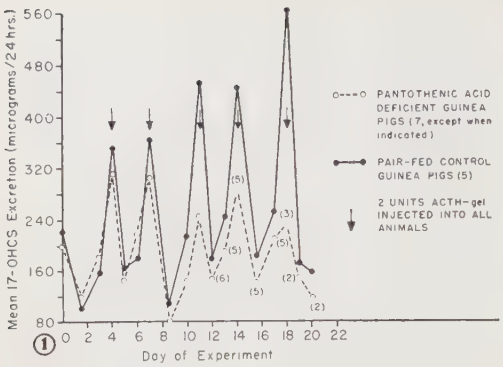
* This investigation was supported by grants from Williams-Waterman Fund and National Vitamin Fn.

were performed on hepatic tissue from each animal by the arsenolysis method(7). In one experiment, concentration of coenzyme A was also determined in adrenal tissue. In preliminary studies, an attempt was made to induce pantothenic acid deficiency by feeding a pantothenate deficient diet previously described by Reid(8). Manifestations of deficiency appeared slowly and animals gained weight for 4 weeks or longer after being placed on the diet. It was also observed that hepatic coenzyme A content of guinea pigs fed this diet for one month was only slightly less than that of control animals, thus demonstrating that severe pantothenate deficiency had not been induced. For this reason, omega-methyl pantothenic acid, a pantothenate antagonist, was incorporated in the diet fed to induce this deficiency in guinea pigs. *Exp. 1*: One group was fed synthetic, pantothenic acid deficient diet(8) containing 0.1% omega-methyl pantothenate. Controls were pair-fed a similar diet to which pantothenic acid was added (40 mg/kg). These animals were divided into 2 subgroups, one of which was fed the control diet without added omega-methyl pantothenate, and the other consumed the same diet containing this antimetabolite. Urinary excretion of adrenocortical hormones by each guinea pig was determined daily except for weekends when determinations were made on a 48-hour sample. Several times during the experiment, ACTH-gel (2 units) was administered subcutaneously to all animals. Guinea pigs of the deficient group were sacrificed when severely ill. When only a few animals survived, those remaining and all guinea pigs in control groups were sacrificed by decapitation. Immediately thereafter, a portion of liver tissue was removed, weighed, homogenized in distilled water, and prepared for subsequent coenzyme A analysis. Tissue homogenates were immediately frozen and kept at −20°C until these analyses were made. *Exp. 2*: One group consumed the pantothenic acid deficient diet containing 0.1% omega-methyl pantothenate while another consumed a similar diet deficient in ascorbic acid which contained no vitamin antagonist. Twenty-four hour urine collections for measurement of 17-hydroxycorticoid content were obtained 4 to 5 times/week and ACTH-gel (2 units) was administered several times. Guinea pigs of both groups were sacrificed when severe manifestations of vitamin deficiencies appeared and hepatic and adrenal tissue were removed for determination of coenzyme A content.

Results. Exp. 1: Guinea pigs fed the pantothenate deficient diet containing omega-methyl pantothenic acid gained weight slowly and seemed in good health for first 10 to 12 days. Subsequently, the animals moved about only when prodded, fur became ruffled, and weight gain ceased. Few guinea pigs survived longer than 3 weeks on this diet. In contrast, pair-fed control animals gained weight and were active throughout the period of observation, regardless of whether or not the diet contained omega-methyl pantothenic acid.

Liver Coenzyme A concentrations were significantly ($P = <0.01$) less in deficient guinea pigs than in control animals which did not consume omega-methyl pantothenate, being 44.0 ± 3.3 and 82.9 ± 5.3 units/g of wet tissue respectively. Since pantothenic acid occurs in the body as integral part of coenzyme A, it is evident that guinea pigs consuming the deficient diet containing omega-methyl pantothenic acid were severely depleted of this vitamin. In control animals feeding of omega-methyl pantothenate did not significantly alter the liver content of coenzyme A.

The mean daily urinary excretion of 17-hydroxycorticosteroids by deficient and control animals is shown in Fig. 1. It is apparent that there was little difference in hormone excretion by the 2 groups on days when ACTH was not administered, although in most instances the value for control guinea pigs was higher than the deficient group. The first 2 times ACTH-gel was given, guinea pigs on pantothenic acid deficient diet exhibited no manifestations of deficiency and steroid excretion by this group was only slightly less than that of controls. As animals on the deficient diet developed signs of depletion, excretion of adrenocortical hormones remained relatively constant while control guinea pigs excreted larger amounts of corticosteroids with each injection of ACTH. Progressive increase in steroid excretion by controls in response to



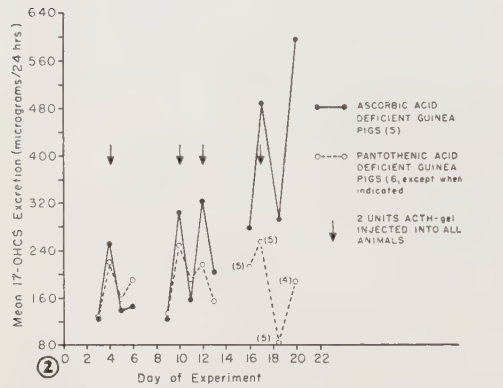


FIG. 1. Mean daily excretion of 17-hydroxycorticosteroid hormones by pantothenate deficient and control guinea pigs.

FIG. 2. Mean daily excretion of adrenocortical hormones by pantothenate deficient and ascorbic acid deficient guinea pigs.

ACTH was statistically significant ($P =< 0.05$) using linear regression and analysis of variance. In the same analysis, the difference between the mean 17-hydroxycorticoid excretion in response to ACTH of deficient and control groups was also statistically significant ($P =< 0.005$).

Exp. 2: Manifestations of pantothenic acid deficiency were similar in all respects to those mentioned in the first study. Animals consuming Vit. C-free diet gained weight slowly for about 13 days following which weight loss and other manifestations of deficiency appeared. Both deficiency states became severe almost simultaneously so that all animals were sacrificed between days 21 and 24 of experiment.

Severe depletion of pantothenic acid occurred in animals fed the diet deficient in this vitamin. Mean liver coenzyme A concentration in pantothenate depleted guinea pigs was 42.8 ± 2.0 units/g wet tissue compared to 97.8 ± 4.0 u/g in scorbutic animals. This is a significant difference ($P =< 0.01$). Mean adrenal coenzyme A levels were also significantly different ($P =< 0.05$), values for pantothenic acid deficient and scorbutic guinea pigs being 52.6 ± 4.8 u/g and 70.9 ± 4.0 u/g respectively. Liver coenzyme A content was somewhat higher in scorbutic guinea pigs than in controls of previous experiment, but this difference was not statistically significant.

Adrenocortical hormone excretion by the 2 groups is presented in Fig. 2. Pantothenate-deficient guinea pigs showed only a slight increase in excretion of these hormones when given ACTH-gel, as observed in previous experiment. Scorbutic animals, however, showed a striking rise in 17-hydroxycorticosteroid excretion following corticotrophin administration. The difference in mean steroid excretion by the 2 groups in response to ACTH was statistically significant, $P =< 0.005$. Excretion of adrenal steroids by scorbutic guinea pigs showed a progressive rise as they became depleted of Vit. C. On the final day when these animals were severely scorbutic, excretion of adrenocortical hormones was greater than at any other time, even though ACTH was not administered. The difference in mean steroid excretion by scorbutic and pantothenate deficient animals on this day was statistically significant, $P =< 0.001$.

Discussion. Excretion of 17-hydroxycorticosteroids in response to ACTH was reduced in guinea pigs fed a pantothenic acid deficient diet containing a pantothenate antagonist. Coenzyme A analyses performed on hepatic tissue from these animals revealed that severe pantothenate deficiency had been induced. Similar results have been observed in pantothenic acid depleted rats(4,5). One aspect which must be considered, however, is that pantothenate deficient guinea pigs were in poor general condition during the latter part of the study while control animals remained well. Thus, during the last few days excretion of adrenocortical hormones by sick animals was compared with that of healthy, though partially starved, guinea pigs and it seemed possible that decreased adrenal ster-

oid secretion might have occurred as a manifestation of illness rather than as a specific result of pantothenate depletion. Although in many nutritional experiments metabolic processes occurring in deficient and, often, sick animals are compared with those in animals that are well, it is desirable to control the factor of illness if possible. For this reason, it was decided to compare the excretion of adrenocortical hormones by pantothenate deficient guinea pigs with that of guinea pigs depleted of another vitamin.

In the second experiment, the 17-hydroxycorticoid excretion of pantothenate depleted animals was compared with that of Vit. C deficient guinea pigs. The decision to use ascorbic acid depleted animals as controls was based on 2 observations. First, other studies have shown that Vit. C deficiency can be induced in young guinea pigs in approximately 3 weeks, the same period required to induce severe pantothenate depletion. Secondly, previous investigation(9) demonstrated that ascorbic acid deficient guinea pigs excrete large quantities of adrenal steroid hormones, even when severe scurvy is present.

Since the purpose of this experiment was to learn whether pantothenate depletion *per se* or illness associated with deficiency resulted in diminished adrenal steroid secretion, it seemed appropriate to use as control nutritional deficiency one in which 17-hydroxycorticosteroid excretion is maintained at high levels. The ascorbic acid deficient group showed a progressive increase in adrenal steroid excretion, even though they demonstrated severe manifestations of vitamin depletion. In addition, a further striking rise in excretion of adrenal hormones occurred each time ACTH was administered. In contrast, excretion of adrenal steroids by the pantothenate deficient group remained relatively constant and was much less than that of the scorbutic group, especially during the latter part of the study when animals in both groups were ill. Coenzyme A content of liver and adrenal tissue confirmed the presence of marked pantothenic acid depletion in guinea pigs fed the diet deficient in this vitamin, but was normal in the scorbutic group.

The data show that illness occurring as a manifestation of nutritional deficiency does not necessarily result in decreased function of the adrenal cortex as measured by 17-hydroxycorticosteroid excretion. It is concluded that diminished adrenocortical hormone excretion which occurs in pantothenic acid deficient guinea pigs is a specific effect of vitamin deficiency and is probably due to diminished body content of coenzyme A, an essential cofactor in the synthesis of steroids, as well as other complex substances.

Summary. 1. Pantothenic acid deficiency was induced in guinea pigs by feeding deficient diet to which omega-methyl pantothenic acid, a vitamin antagonist, was added. 2. Urinary excretion of adrenocortical hormones by pantothenate depleted guinea pigs was compared with that of pair-fed controls and was significantly reduced. 3. Excretion of 17-hydroxycorticosteroids by pantothenic acid deficient guinea pigs was significantly decreased when compared with that of ascorbic acid depleted animals. 4. Diminished adrenocortical hormone secretion which occurred in these animals was a specific effect of pantothenic acid depletion and not a non-specific manifestation of nutritional deficiency.

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Study of Hydrolysis of Urinary Metabolites of 2-Methyl-1,4-Naphthoquinone. (23897)

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Several investigators have measured the concentration of urinary metabolites of 2-methyl-1,4-naphthoquinone after administration of both radioactive(1-4) and non-radioactive(5-7) forms of this quinone. Since these metabolites have been detected only in conjugated forms, hydrolysis has been a necessary prerequisite to quantitative determination of the non-radioactive compound. Agents used for hydrolysis of these conjugates include enzymes, ceric sulfate, hydrochloric acid, and a combination of stannous chloride and hydrochloric acid. Different recoveries of administered 2-methyl-1,4-naphthoquinone have been obtained by investigators. These variations may be due to differences in hydrolytic procedures employed or in species studied. Richert reported that rabbits excrete 31-42% in forms hydrolyzed by hydrochloric acid(5), and an additional 25-30% liberated by ceric sulfate(6). Rat urine collected 24 hours following injection of 2-methyl-1,4-naphthoquinone was studied for its content of metabolites. Canady(7) demonstrated 50% in a form hydrolyzed with hydrochloric acid alone or in presence of stannous chloride. Li(1) found 13-20% after treatment with hydrochloric acid or bacterial β-glucuronidase. Forty-five % of radioactivity of C^{14} labeled 2-methyl-1,4-naphthoquinone was recovered by Jaques(4). Three conjugates, phosphate, diglucuronide, and monosulfate have been separated by chromatographic methods from urine collected following administration of radioactive 2-methyl-1,4-naphthoquinone(3).

In our investigation the conjugates of 2-methyl-1,4-naphthohydroquinone present in urine of rats were separated by paper chromatography. Two types of urine containing these conjugates were chromatographed: 1) untreated urine and 2) urine remaining after treatment with specific hydrolytic agent. After their chromatographic separation they were hydrolyzed with ceric sulfate and with hydrochloric acid. These procedures permitted estimation of relative concentration of each conjugate and the response of conjugates to different hydrolytic procedures.

Methods. Urine collection. Urine was collected under petroleum ether (B.P. 40-50°C) at 0°C for 24-hour periods from 6 groups of 3 rats each. During these periods, rats were housed in metabolism cages with raised wire floors and subfloors to prevent contamination of urine with feces. They had access to water and ground rat chow *ad libitum*. Examination of such urine for its content of metabolites of 2-methyl-1,4-naphthoquinone was conducted in the following sequence: 1) Urine was collected from normal rats since such urine contains products which influence colorimetric determination of 2-methyl-1,4-naphthoquinone. They were chromatographed, hydrolyzed and measured quantitatively. Values obtained were considered as blanks for each individual rat, and were subtracted to give a true value for 2-methyl-1,4-naphthoquinone present in experimental urine. 2) Urine was collected following administration of 2-methyl-1,4-naphthoquinone. To obtain high urinary concentrations of metabolites this quinone was administered at 11 mg/kg to normal male rats weighing 300-400 g. Vitamin, dissolved in 1 ml of dibutyl succinate was injected subcutaneously into the left hind leg. Urine was examined qualitatively and quantitatively for content of metabolites of 2-methyl-1,4-naphthoquinone. Qualitatively, the conjugates in urine were separated by paper chromatography and isolated conjugates hydrolyzed by hydrochloric acid and by ceric sulfate. Chromatograms were prepared from untreated urine as well as from urine remaining after hydrolysis by procedures indicated below. Quantitatively, a determination was made of 2-methyl-1,4-naphthoquinone liberated following hydrolysis of urine with 1. hydrochloric acid, 2. ceric sulfate, 3. hydrochloric

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acid followed by ceric sulfate, and 4. Ketodase.† It has been demonstrated previously (8) that treatment of normal rat urine and aqueous solutions containing added 2-methyl-1,4-naphthoquinone by hydrolytic and extraction procedures described here permitted a recovery of 82-88%. *Chromatography.* Conjugates of 2-methyl-1,4-naphthohydroquinone were separated by the ascending technic of Hoskin(3) on Whatman No. 1 paper, 17″ x 22½″. A 1 ml aliquot of urine was deposited on a line 9 inches long and 2 such bands were placed on each sheet, one from untreated urine, the other from urine after hydrolysis. Chromatograms were developed in the solvent system containing butanol, methanol, water and acetic acid until the solvent front traveled about 35 cm from origin. After drying in air, the papers were examined with an ultraviolet lamp and from areas of fluorescence and absorption, the positions of conjugates of 2-methyl-1,4-naphthohydroquinone were determined. In each experiment 2 sheets were developed simultaneously. After separation, all conjugates on one sheet were treated with hydrochloric acid, all on the other with ceric sulfate. Hydrolytic procedures were applied either to urine or to paper containing conjugates previously separated by chromatography. When urine was used a volume was selected which would yield approximately 100 μg of 2-methyl-1,4-naphthoquinone following hydrolysis, and this quantity was diluted to 10 ml. Hydrolysis of compounds separated by paper chromatography was effected by cutting the filter paper containing a particular conjugate into 10 ml of water. Urinary conjugates were hydrolyzed by the following agents: A. *Hydrochloric acid.* Ten ml of urine and 1 ml of concentrated hydrochloric acid were placed in flask attached to water-cooled West condenser and the solution boiled 30 minutes. The condenser and its fittings were then washed with petroleum ether and the washings added to the reaction mixture. B. *Ceric Sulfate.* To 10 ml of urine overlaid with petroleum ether were aded 2 ml of glacial acetic acid and 5 ml of a solution of 0.1 N ceric sulfate in 1 N H_2SO_4. This mixture was allowed to stand 5 minutes at room temperature and was then diluted and extracted. C. *Hydrochloric acid followed by ceric sulfate.* Ten ml of urine were hydrolyzed with hydrochloric acid as described above. Hydrolysis with ceric sulfate was then carried out by adding 2 ml of glacial acetic acid and 5 ml of ceric sulfate solution and allowing the mixture to stand 5 minutes at room temperature. D. *Ketodase.* Ten ml of urine at pH 7.0 were placed in flask attached to water-cooled West condenser and heated 30 minutes in boiling water to inactivate urinary enzymes. The solution was cooled to room temperature and the condenser and its fittings were washed with petroleum ether and the petroleum ether washings added to the solution in the flask. pH was adjusted to 4.5 and Ketodase, assayed by the method of Talalay(9), was added at a level of 400 units of β-glucuronidase/ml followed by 0.2 ml of an 80 mg % solution of 8-hydroxyquinoline. Urine was incubated 24 hours at 37°C. *Extraction.* After hydrolysis contents of flask were transferred to a separatory funnel using such volumes of water and petroleum ether that final volumes for extraction were 50 ml of each solvent. The aqueous mixture was extracted twice with 50 ml portions of petroleum ether. The combined petroleum ether extract was washed once with 2 ml of 0.5% $NaHCO_3$ solution and once with 1 ml of water. The volume of petroleum ether was reduced under diminished pressure and last traces were carefully removed by evaporation under stream of nitrogen. The residue was immediately taken up in 1 ml of absolute ethanol. *Assay.* 2-methyl-1,4-naphthoquinone was determined by modification of the procedure of Richert(5). The ethanol solution was transferred to test tube calibrated to contain 6 ml using borate buffer pH 10.5 to complete the transfer. Three drops of ethyl cyanoacetate were added and 10 minutes allowed for color development. Pigments which were extracted from urine with petroleum ether were in this solution and could be almost completely removed by successive extractions with 2 ml portions of petroleum ether and diethyl ether. The solution obtained by application of these technics

† Obtained from Warner-Chilcott Laboratories, N. Y.

TABLE I. Urinary Excretion of 2-Methyl-1,4-naphthoquinone by the Normal Rat.

Method of hydrolysis	Conc. of 2-methyl-1,4-naphthoquinone, μg/ml
HCl	109
$Ce(SO_4)_2$	95
Ketodase	102
HCl followed by $Ce(SO_4)_2$	123

These values represent conc. of this quinone in urine collected for 24 hr following administration of 2-methyl-1,4-naphthoquinone at level of 11 mg/kg.

to paper chromatograms was filtered through a sintered glass funnel. Optical density of final solution was read in Beckman D.U. spectrophotometer at 570 mμ.

Results. Hydrolysis of urine containing metabolites of 2-methyl-1,4-naphthoquinone release this quinone in amounts which were determined by method employed. As indicated in Table I treatment with hydrochloric acid yielded greater amounts (109 μg/ml) than did hydrolysis with ceric sulfate (95 μg/ml). When hydrochloric acid treatment was followed by ceric sulfate 123 μg/ml were released.

Phosphate, glucuronide and sulfate were separated chromatographically from rat urine containing metabolites of 2-methyl-1,4-naphthoquinone. As seen in Table II ceric sulfate and hydrochloric acid were not equally effective in hydrolyzing these conjugates. Boiling in hydrochloric acid liberated more 2-methyl-1,4-naphthoquinone from sulfate than was released after treatment with ceric sulfate, the latter being about 78% as effective as acid in hydrolyzing the sulfate. Ceric sulfate liberated more 2-methyl-1,4-naphthoquinone from the glucuronide and phosphate than was obtained after treatment of these conjugates with hydrochloric acid. In hydrolysis of the glucuronide, hydrochloric acid was 89% as effective as ceric sulfate. A greater difference existed in ability of these reagents to split phosphate, with acid only about 55% as effective as ceric sulfate.

Using data obtained from hydrolyses of these conjugates, estimates were made of their distribution in urine. Hydrolysis with ceric sulfate suggested the following: 22% phosphate, 46% glucuronide, 32% sulfate. After hydrolysis with hydrochloric acid 12% phosphate, 44% glucuronide and 44% sulfate were obtained. These figures indicate that 2-methyl-1,4-naphthohydroquinone was conjugated primarily as glucuronide and to least extent as phosphate.

Chromatograms were also prepared from urine remaining after treatment with the hydrolytic agents. Examination of these chromatograms indicated that conjugated 2-methyl-1,4-naphthohydroquinone was still present. In data summarized on Table III it can be seen that following hydrolysis with hydrochloric acid or with ceric sulfate significant quantities were present in the compound having the R_f of sulfate. Little phosphate and glucuronide remained after treatment with either of these agents. When hydrolysis was accomplished by a combination of hydrochloric acid and ceric sulfate almost all of conjugated hydroquinone was removed. The source of this additional quinone might be inferred from examination of values in Table III. These data suggest that sulfate, present in urine after its hydrolysis with hydrochloric acid was removed from urine which had been hydrolyzed with both hydrochloric acid and ceric sulfate. Doubling the hydrolysis time or concentration of hydrochloric acid did not result in an increased release of 2-methyl-1,4-naphthoquinone from urine. Incubation of

TABLE II. Concentration of 2-Methyl-1,4-naphthoquinone in Conjugates Separated from Normal Rat Urine.

	Conjugate	Method of hydrolysis: $Ce(SO_4)_2$ (μg/ml)	Method of hydrolysis: HCl (μg/ml)
	Sulfate	13.1	18.9
	Glucuronide	21.1	19.4
	Phosphate	11.2	3.9
	Sulfate	13.4	18.8
	Glucuronide	20.5	19.1
	Phosphate	8.7	4.4
	Sulfate	15.3	17.9
	Glucuronide	20.5	18.5
	Phosphate	10.6	6.1
	Sulfate	15.9	18.4
	Glucuronide	21.7	16.8
	Phosphate	8.6	7.3
Avg	Sulfate	14.4	18.5
	Glucuronide	20.9	18.5
	Phosphate	9.8	5.4

Each value above represents avg of 3 determinations.

TABLE III. Effect of Various Hydrolytic Procedures on Urinary Metabolites of 2-Methyl-1,4-naphthoquinone.

Method of hydrolysis	Conjugate	Original urine		Urine after hydrolysis	
		Method of hydrolysis		Method of hydrolysis	
		$Ce(SO_4)_2$	HCl	$Ce(SO_4)_2$	HCl
		μg/ml			
HCl	Sulfate	12.9	16.2	17.9	23.9
	Glucuronide	24.4	25.0	.8	.4
	Phosphate	5.5	2.7	.8	.6
$Ce(SO_4)_2$	Sulfate	14.5	19.7	8.2	12.3
	Glucuronide	21.2	20.0	1.5	0
	Phosphate	7.7	4.8	3.3	1.1
Ketodase	Sulfate	17.0	19.5	6.5	7.0
	Glucuronide	21.3	21.2	15.0	11.4
	Phosphate	10.5	7.7	13.5	7.3
HCl followed by $Ce(SO_4)_2$	Sulfate	13.2	14.5	4.7	2.1
	Glucuronide	16.2	14.4	3.7	0
	Phosphate	10.1	7.8	1.9	1.7

urine with Ketodase resulted in removal of small quantities of glucuronide and greater quantities of sulfate. This enzyme preparation did not hydrolyze the phosphate although it contains both an acid and alkaline phosphatase.

Summary. Urinary metabolites of 2-methyl-1,4-naphthoquinone have been separated by paper chromatography and their response to various hydrolytic procedures studied. Three conjugates, phosphate, glucuronide and sulfate were hydrolyzed both by hydrochloric acid and by ceric sulfate. The sulfate was hydrolyzed to a greater degree by hydrochloric acid and this reagent was less effective than ceric sulfate in splitting phosphate and glucuronide. Following treatment of rat urine with hydrochloric acid or with ceric sulfate, significant quantities of sulfate remained unhydrolyzed. A combination of these procedures hydrolyzed the sulfate. 2-methyl-1, 4-naphthoquinone was released from glucuronide and sulfate after incubation in the presence of Ketodase. The conjugate present in lowest relative concentration was phosphate, that present in largest amount was glucuronide.

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Thromboplastic and Fibrinolytic Activity of Human Synovial Membrane and Fibrous Capsular Tissue.* (23898)

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The balance between clot formation and fibrinolysis regulates local hemostasis(1). We have therefore investigated the thromboplastic and fibrinolytic activities in the human synovial membrane and fibrous capsular tissue to elucidate their role in intra- and periarticular hemorrhage.

Materials and methods. Thromboplastic activity: Fresh tissue (100 mg) was suspended in 0.9% NaCl (0.90 ml) in a Potter homogenizer and stored at –20°C for a few days. After thawing and retreatment in the homogenizer, coarse particles were removed by filtration through cotton wool. Serial dilutions were prepared and clotting times (at 37°C) estimated in mixture consisting of 0.2 ml fresh rabbit citrate plasma, 0.5 ml 0.9% NaCl and 0.1 ml of tissue suspension, after recalcification with 0.2 ml 0.030 M $CaCl_2$ solution. For comparison the activity of a suspension of human brain (as used in Owren's P-P method for estimation of prothrombin activity) was simultaneously estimated. *Fibrinolytic activity*. The amount of plasminogen activator in tissues was estimated according to the principle recently described (2), but with minor modifications since the activities were low and a quantitative extraction of the active principle was not required. Fresh tissue (100 mg) was thoroughly disintegrated in a Potter homogenizer with 2 M KSCN (3.0 ml) followed by slow shaking for 2 hours. After centrifugation the supernatant was diluted with 7 volumes of H_2O and N HCl was added until pH 1 was obtained. After standing for 15 minutes and centrifuging, the precipitate was redissolved in 1 ml 2 M KSCN by neutralization (litmus paper) with solid $NaHCO_3$. This solution was 3 times concentrated with respect to original solution. In some cases, the high lipid content of the tissue sample prevented complete sedimentation by centrifugation and the suspension obtained after first centrifugation was used without additional acid precipitation. Activity was estimated by the fibrin plate method(3) (0.1% bovine fibrinogen) and compared with standard preparation of plasminogen activator from pig heart tissue as previously described (2). *Tissues*: Samples of synovial membrane and of fibrous capsular tissue were obtained from knee joints of 10 persons dying from diseases unrelated to the joints.

Results. None of the samples investigated showed more than a trace of *thromboplastic activity*. Most samples of fibrous tissue had no activity, the rest only traces, and this result could not be caused solely by incomplete dispersion of the fibrous tissue. The synovial tissue was easily dispersed but nonetheless showed only traces of thromboplastic activity. This is demonstrated in Fig. 1, typical of the whole series. It is seen that synovial tissue has only a few per cent of activity of brain suspension used for comparison.

All samples showed *fibrinolytic activity* caused by a tissue activator of plasminogen. This is a stable activator different from the activator found in blood(4). Fig. 2 is an example of an estimation. By interpolation on the standard curve the concentration is 180 units/g synovial tissue and 60 units/g of fibrous tissue. This is in the low (or medium) range of activities found in human organs(5). There were great individual variations, similar to those observed previously (5,6). As mentioned, difficulties were encountered in some estimations. The following additional values were all obtained after the complete procedure (units/g): Synovial tissue: 63, 57, 46, 25, 185; Fibrous tissue: 25, 25, 35, 12, 114.

Discussion. Since no or only slight throm-

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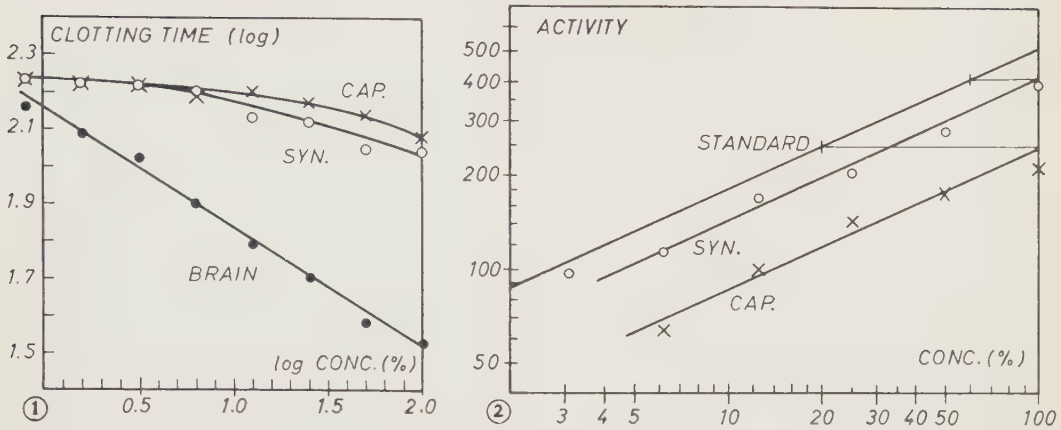


FIG. 1. Thromboplastic activity of human synovial tissue (Syn.) and fibrous capsular tissue (Cap.). *Abscissa:* Logarithm of conc. (in % of stock solution). *Ordinate:* Logarithm of clotting time (sec.). A standard preparation of human brain is used for comparison.

FIG. 2. Plasminogen activator in human synovial tissue (Syn.) and fibrous capsular tissue (Cap.). (Same patient as in Fig. 1.) *Abscissa:* Logarithm of conc. (in % of stock solution). *Ordinate:* Logarithm of activity (product of diameters in mm² of lysed zones). A standard preparation of pig heart is used for comparison (30 units/ml).

boplastic activity is present in joint tissues (synovial and fibrous capsular tissue) it is apparent that local hemostasis, after injury of a joint, must be effected chiefly by activation of the plasma thromboplastin system. Subsequent removal of clotted fibrin can then be caused by an effect of the plasminogen activator found in the tissues. This explains why, after hemorrhage in a joint, blood can normally be emptied in fluid condition after a few days.

The high latent activity of the plasma thromboplastin system in normal persons secures normal clotting and hemostasis even at sites where the amount of tissue thromboplastin in the injured tissue is low. In patients with hemophilioid disorders, especially classical hemophilia (AHF deficiency) and Christmas disease (PTC deficiency), bleedings in joints are characteristic symptoms. This can now be easily explained by lack of thromboplastic activity in joint tissues together with deficiency in plasma thromboplastin system occurring in these patients.

Since the defective plasma thromboplastin system can prolong extravasation of blood at sites where the amount of tissue thromboplastin released from injured tissue is low, this can give rise to increased formation of clots which are difficult to dissolve completely again by available amount of fibrinolytic agents. After organization the final result would be a formation of excess of connective tissue. The frequent occurrence of crippling of joints after hemarthrosis in hemophilic patients is an example of this sequence of events, and is in good agreement with experimental findings reported here.

As pointed out recently(7), occurrence of arteriosclerotic lesions in hemophilic patients can be explained on the basis of balance between fibrin deposition and fibrinolysis. The results presented here demonstrate the usefulness of this concept in explaining pathological processes(1).

Summary. 1. Human synovial tissue and fibrous capsular tissue contain no or only traces of tissue thromboplastin. An activator of plasminogen of the tissue type is present in low (but varying) concentrations in both tissues.

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Growth in Tissue Culture of Cytopathogenic Agent from Strain of Virus which Produces Avian Lymphomatosis. (23899)

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Many attempts have been made to propagate in tissue culture, viruses which cause several neoplasms in the fowl. The majority of studies were performed with several forms of avian leukosis(1-3). Early studies were concerned with cultivation of virus from explanted neoplastic tissue. These attempts at serial passage for several generations, in tissue culture, have failed. Recent studies(4,5) indicate that the Rous sarcoma virus can be successfully cultivated in chick fibroblast cultures, often producing distinct cellular changes. Preliminary observations on growth of visceral lymphomatosis (V.L.) virus in tissue cultures have been reported(6,7). The greatest difficulty is the long latent period of disease in the host(8). Thus, any studies involving biological expressions of the virus may take as long as 9 months. Attempts to cultivate the virus in a host other than chicken have generally failed. It has been reported (9) that V.L. virus can be serially propagated in embryonating chicken egg; however, no gross or microscopic lesions are observed in the embryo. This paper describes recovery and cultivation of a cytopathogenic agent from strain RPL 12 which not only causes visceral lymphomatosis, but under certain conditions also erythroblastosis, osteopetrosis and, sporadically, still other neoplasms.

Materials and methods. Virus. Inoculum L29 was derived from pool of 5 livers from chickens of 17th serial cell-free passage of strain RPL 12. Inoculum L30 was from liver of one chicken of 15th serial passage of same strain. Inoculum L31 was from a pool of 11 livers from chickens of 16th serial passage. Recovery of virus and preparation of cell-free inoculum have been described(8). *Sera.* Antiserum against tissue culture propagated virus was prepared in adult chickens by injecting into breast muscle a virus-adjuvant mixture 4 ml/injection, in 2 vaccinations spaced 3 weeks apart. Normal chickens from isolated population were used for preparing all chicken anti-RPL 12 serum, as well as supplying a source of normal serum. Antiserum produced in rabbits with tissue cultured strain RPL 12 virus was kindly supplied by Dr. G. R. Sharpless.* All sera were treated at 56°C for 30

TABLE I. Growth and Cytopathogenicity of Virus from Strain RPL 12 Visceral Lymphomatosis.

Passage No.	Total effective dilution, negative logs			$TCID_{50}$, negative logs			Latent period of CPE		
	L29	L30	L31	L29	L30	L31	L29	L30	L31
1	2	2	2					5 da	48 hr
2	3	3	3			5.6		3 "	18–24 "
3	4	4	6	4.0		7.0	5 da	"	"
4	5	5	10		5.0	7.0	"	"	"
5	6	6		5.0			"	48 hr	
6	7	7			5.0			"	
7	8	8		8.0	6.0		48 hr	24 hr	
8	9	9					"	"	
9	10			8.0			"		
10	11			7.5			18–24 hr		
11	15			7.0			"		
12	19			7.3			"		
13	24			7.1			"		

* Virus and Rickettsial Research Div. Am. Cyanamid Co., Pearl River, N. Y.

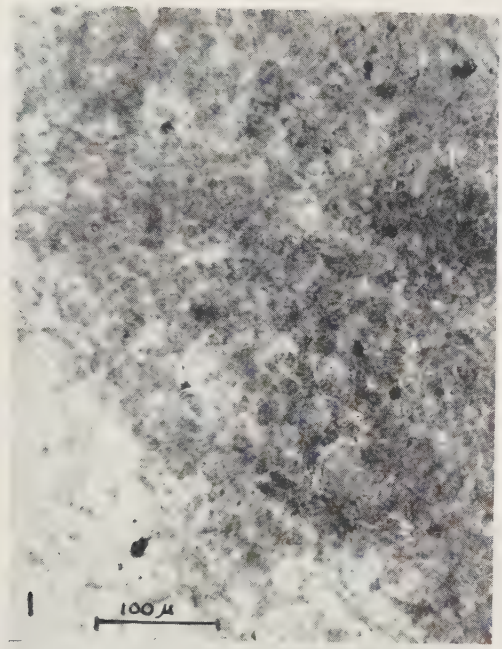




FIG. 1. Uninoculated culture of chicken embryo liver.

FIG. 2. Cytologic changes in embryo liver culture 24 hr after inoculation with strain RPL 12 virus. Note degeneration in epithelial-like cells.

minutes prior to use. *Tissue cultures.* Livers from 14-day chick embryos were minced and trypsinized by method similar to that of Youngner(10). Tubes were seeded with 0.8 ml of 1:200 dilution of cells in Mixture 199 (11) containing 0.14% sodium bicarbonate and 30% horse serum. The tubes were incubated at 37°C. A confluent monolayer was formed in about 3 days and growth medium was replaced with 199 alone or 199 plus 25% Difco Tryptose Broth and 2% horse serum. Passage and bioassay were made by replacing maintenance media with 0.8 ml virus at appropriate dilutions.

Results. 1. *Recovery and passage of virus.* Cultures were observed daily for 7 days, during which time no cytopathogenicity was observed. A 2nd blind passage was made and cytopathogenic effect (CPE) was again inapparent at 7th day. In passage 3 CPE became apparent on the 5th day in 5 of 12 cultures. Fluids from these 5 tubes were pooled for the next passage. The virus was carried through 10 consecutive passages. Beginning with passage 11, cultures showing CPE at the terminal endpoint dilution were used for the next passage. At passage 13 the original virus inoculum would have been theoretically diluted to 10^{-24}. Data relative to the serial passage of the different preparations of RPL 12 are given in Table I. In general, cellular changes followed a definite sequence; refractile granules appeared in the cytoplasm of the polygonal epithelial-like cells; the nucleus became pyknotic, and this was followed by shrinkage of cytoplasm and rounding up of the cells, together with a tendency toward formation of grape-like clusters. At approximately 2 days after inoculation, the clusters began to slough off the glass. The spindle shaped cells remained viable up to the 5th or 6th day when a CPE became evident manifested by margination of chromatin and karyorrhexis. Comparison of normal and infected chicken embryo liver tissue cultured cells can be seen in Fig. 1, 2, and 3.

2. *Pathogenicity of tissue culture virus for chicks.* Three groups of chicks were inoculated intravenously at 2 weeks of age with 0.2 ml tissue culture virus. Of 22 chicks inoculated with fluids from the 2nd passage, 13 died showing typical lesions of visceral lymphomatosis. Of 25 chicks inoculated with fluids from the 3rd passage, 13 died of visceral lymphomatosis, and 2 of erythroblastosis.

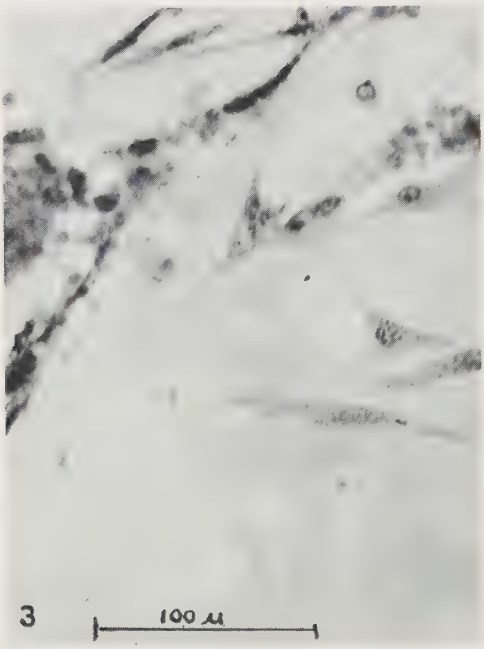


FIG. 3. Cytologic changes in embryo liver culture 96 hr after inoculation. Note clumping of chromatin in spindle-shaped cells.

Chicks inoculated with fluids from passage 10 responded with only 6 out of 50 birds developing visceral lymphomatosis. In 3 groups of chickens death occurred between 117 to 260 days postinoculation. These results are typical of response in chickens to virus freshly isolated from lymphomatous livers (8), where a proportion of chickens are always refractive.

3. *Serum neutralization tests.* Serum or serum dilutions were mixed with equal volumes of tissue culture fluids in final concentrations of 100 or 1000 $TCID_{50}$ of virus. The serum-virus mixtures were incubated 2 hours at 20°C and 0.8 ml inoculated into each of 10 tubes. Tests were concluded when cells in the virus control tubes showed complete CPE. Serum from a chicken which had been hyperimmunized with virus from the 10th tissue culture passage, diluted 1:256, neutralized 1000 $TCID_{50}$ of virus, while serum from the same chicken taken before immunization, diluted 1:16, failed to neutralize the virus. Rabbit anti-tissue culture-virus serum at 1:256 dilution also neutralized 1000 $TCID_{50}$ of virus. These results show that the virus was capable of stimulating specific neutralizing

TABLE II. Neutralization of Tissue Culture RPL 12 Virus by V. L. Antisera from Various Sources.

Serum	Source	Serum dilution neutralizing 1000 $TCID_{50}$
Chicken anti-TCP	Individual hen	1: 256
Idem	Pooled	"
Rabbit anti-TCP	Individual rabbit	"
Chicken anti-CP	Pooled	1: 128
Idem	Individual hen	1:1024
"	Pool of progeny from vaccinated hens	1: 64
Normal	Pool of progeny from unvaccinated hens	0
"	Pool from isolated hens	0

TCP = Tissue culture virus. CP = Chicken virus.

antibodies. Antiserum to strain RPL 12 chicken tumor produced in chickens, likewise showed neutralizing properties against tissue culture passed virus, as did serum from progeny of RPL 12 immunized hens. Details for this immunization procedure have been described(12). Serum pools from an isolated population, at 1:4 dilution, failed to neutralize 100 $TCID_{50}$ of virus. Results of these neutralization tests shown in Table II indicate that the cytopathogenic agent for chick embryo liver cell culture is related, at least antigenically, to chicken propagated RPL 12 virus.

Discussion. Evidence has been presented which indicates that a cytopathogenic agent for chick embryo liver tissue culture was recovered from filtrates of leukotic livers of chickens inoculated with strain RPL 12 virus. Fluids from serial passages of the virus have induced typical visceral lymphomatosis in inoculated chickens. In tissue culture the virus has shown titers up to 10^{-8} $TCID_{50}$. It is interesting to note that there was a decrease in virulence for the chicken after continuous *in vitro* passage. This is not a peculiar event, since it is well known that viruses isolated from various passages vary in their pathogenicity. Furthermore, viruses grown under different conditions often become modified in their pathogenicity. This can be manifested in partial to complete loss in virulence. The most practical use of this phenomenon has been in the development of modified vaccines.

Additional evidence for relationship of this

cytopathogenic agent to strain RPL 12 virus is provided by results of serum neutralization tests. Antibodies produced in chickens with RPL 12 virus readily neutralized the tissue cultured virus in the same range as did the specific antibodies to tissue cultured virus. A significant fact is that antiserum to a virus of similar origin and biological characteristics, but propagated *in vitro* at another laboratory, neutralized our tissue cultured virus at high dilutions.

Summary. A cytopathogenic agent isolated from RPL 12 in chicken embryo liver tissue cultures is reported. The virus produces a marked CPE on liver parenchymal cells. Virus serially passed in tissue culture, when inoculated into susceptible chicks, produced visceral lymphomatosis. Neutralization tests with antiserum against RPL 12 indicate a relationship of tissue culture virus with RPL 12; however, positive identity of the cytopathogenic agent with the virus causing visceral lymphomatosis has not been established.

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Structural Analogues of Puromycin in Production of Experimental Nephrosis in Rats.* (23900)

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Sherman, Taylor, and Bond(1) first demonstrated that the antibiotic 6-dimethylamino-9 - (3′-p-methoxy-L-phenylalanylamino-B-d-ribofuransoyl) purine (Puromycin) obtained from *Streptomyces alboniger,* has a nephrotoxic effect in rats. Subsequently, it was shown by Frenk and Antonowicz(2), that the derivative of Puromycin, 6-dimethyl-aminopurine-3′-amino - d - riboside (Aminonucleoside), produced a typical nephrotic syndrome. Feigelson, Drake, and Recant(3) described the syndrome in detail. The present work was undertaken to determine the structural configuration of these compounds essential for production of the nephrotic syndrome. To achieve this end, a variety of structural analogues of Puromycin was studied.

Materials and methods. Fifty-five male Sprague-Dawley rats, weighing 80-100 g, were divided into 10 experimental groups of 4 animals, and one control group of 15 animals. Each group was treated with daily subcutaneous injections of 0.3 ml of 0.017 M solution of one of the following: Aminonucleoside, Puromycin, 3-amino - d - ribose, 6-dimethyl-amino-purine, and 6-dimethyl-adenosine. 3′-amino-adenosine was administered in dosages of 0.3 ml of 0.017 M, 0.0085 M, 0.0034 M, and 0.0017 M solutions.† An additional group re-

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† We are indebted to Lederle Laboratories, who generously supplied these compounds.

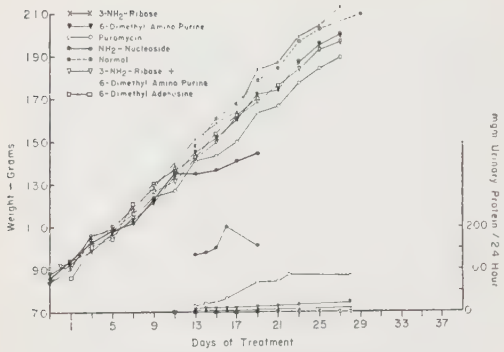


FIG. 1. Weight and proteinuria curves in animals treated with Puromycin analogs.

ceived simultaneous injections of 3-amino-d-ribose and 6-dimethyl-aminopurine. Control animals received no injections since previous controls injected daily with saline or distilled water showed no abnormalities. Animals were maintained on Purina Laboratory Chow and drinking water *ad libitum,* and housed in individual metabolism cages. Observations ranged from 10 to 30 days. Body weights were recorded every second day. To assess activity of the drugs, those constituents of serum and urine characteristically altered in nephrosis were measured. 24-hour urinary outputs were collected and urinary proteins determined by the Esbach method(4). At conclusion of each experiment, the rats were sacrificed by decapitation and blood collected in heparinized tubes. Serum cholesterol was determined on these samples by the method of Saifer and Kammerer(5); total serum protein and albumin and globulin fractions were determined by the micro-method of Lowry, Rosebrough, Farr, and Randall(6), using Howe fractionation technic(7). Kidneys were fixed in Bouin's solution for histologic study.

Results. As seen in Fig. 1, each group of animals showed a weight curve that paralleled the normal, with exception of those being treated with Aminonucleoside. The weight curve of Aminonucleoside-treated animals followed the normal for first 11 days of injection, but at this point the curve became almost flat for 4 days, after which it began to rise again. The secondary phase of weight gain occurred when the animals manifested the accumulation of ascites. The weight curve of Puromycin-treated animals, although somewhat lower than the normal curve, remained essentially parallel to it, and showed no distinct plateau.

Significant proteinuria became manifest in the Aminonucleoside group when growth reached a plateau. Animals receiving Puromycin showed a slowly progressing proteinuria beginning on thirteenth day and reaching a maximum on twenty-second day; the level of proteinuria reached its peak, therefore, at a much later date than in the Aminonucleoside group, and the maximal level was considerably lower. None of the other groups studied showed any significant proteinuria.

As to serum constituents (Table I) it can be seen that only Aminonucleoside animals developed nephrosis as manifested by elevated serum cholesterol, decreased total serum proteins and marked hypoalbuminemia. Serum chemical changes in the Puromycin group were in the same direction, but of much lesser degree. All other animals had normal serum cholesterol and protein values. Table I also reveals that all animals treated with Aminonucleoside had severe ascites at necropsy, whereas animals treated with other drugs had ascites. Microscopic examination of kidneys of the Aminonucleoside animals showed Periodic-Acid-Schiff positive casts, tubular dilatation and widening of Bowman's space; there were no structural changes in the glomeruli. No abnormalities were noted in kidneys of Puromycin-treated animals.

3′-amino-adenosine, although differing from the Aminonucleoside only by absence of dimethyl-substitution on the amino group in position 6 of purine ring (Fig. 3), proved to be considerably more toxic in terms of growth and survival. Animals which received this drug in a dosage equivalent to that used of other drugs showed a rapid loss of weight and died within a few days (Fig. 2). Those which received half this dose also showed rapid weight loss. Indeed, animals treated with only one-tenth the full dose showed average weight gain of only 20 g in 10 days, as compared to 50 g by normal rats. There was no evidence of nephrosis in any animal treated with this compound, *i.e.,* no proteinuria, ascites, hypercholesterolemia, or hypoalbuminemia. Although total serum proteins were moderately depressed, this may be attributed to their extreme state of cachexia. Both gross

TABLE I. Mean Concentration of Certain Constituents of Serum.

Exp. groups	Cholesterol, mg %	Total serum proteins	Albumin	Globulin	Ascites, 0 - ++++
		g %			
Aminonucleoside*	868	3.1	.91	2.19	++++
Puromycin	280	3.75	1.5	2.25	0
6-Dimethylaminopurine	98.5	4.4	2.3	2.1	0
3-Amino ribose hydrochloride	100	4.4	2.3	2.1	0
3′-Amino-3′-deoxyadenosine	103	3.8	2.3	1.5	0
6-Dimethylaminopurine + 3-amino ribose hydrochloride	79.4	5.1	2.6	2.5	0
6-Dimethyl adenosine	99.4	5.2	2.5	2.7	0
Controls	100.9	4.8	2.7	2.1	0

* All compounds were used as 0.017 M solutions except 3′-amino-3′-deoxyadenosine, which was 0.0085 M.

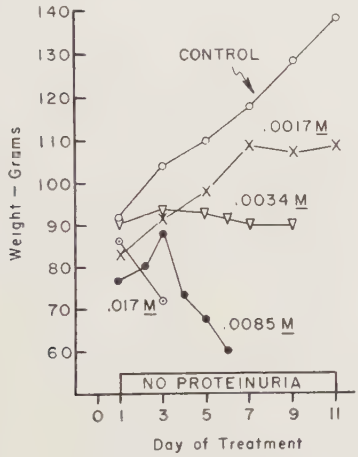


FIG. 2. 3′-amino-adenosine treated animals.

and microscopic examination of kidneys and other major organs failed to reveal any abnormalities.

Discussion. Structural analogues of Puromycin illustrated in Fig. 3 have been tested with reference to their ability to induce the nephrotic syndrome in rats. It is apparent that only 2 of them, Aminonucleoside and Puromycin, are active in this respect (Table I). The milder degree and delay in time of onset of nephrosis, which results from Puromycin as compared to Aminonucleoside, may possibly be related to the complex side chain binding the amino group of Puromycin (Fig. 3). It seems reasonable to assume that before Puromycin is fully effective in production of nephrosis, the side chain must be cleaved from the molecule leaving free Aminonucleoside.

Aminonucleoside differs from the naturally occurring metabolite, adenosine, in 2 respects: 1) presence of a dimethyl substitution on the amino group attached to carbon 6 of the purine ring, and 2) substitution of an amino group on carbon 3 of the ribose moiety. The fact that neither 6-dimethyl-adenosine nor 3′-amino-adenosine, each of which contains one but not the other of these two substitutions, can induce nephrosis, indicates that presence of both substitutions is essential for induction of the syndrome. It is of interest that neither 6-dimethyl-adenine nor 3-amino-ribose, when administered separately or simultaneously, were nephrotoxic. This indicates that although the purine and pentose components of the Aminonucleoside molecule are injected to-

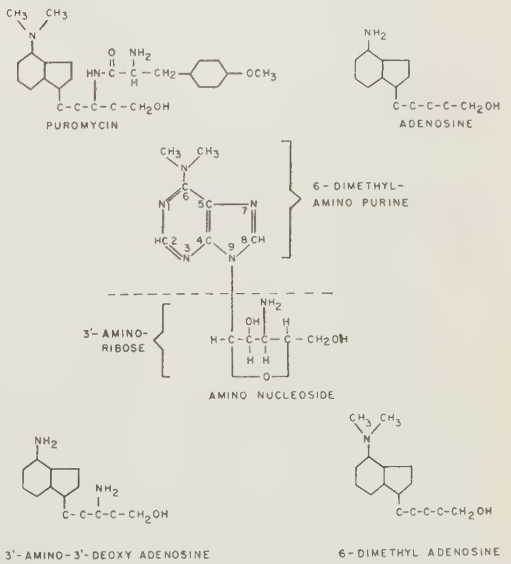


FIG. 3. Chemical structure of Puromycin and its analogs.

gether, they are not conjugated *in vivo* to form the Aminonucleoside in any significant amount.

The striking specificity of structural configuration that is required for production of this drug-induced nephrosis suggests an enzymatic mechanism of action of Aminonucleoside. Evidence to support this contention is being sought.

Summary. Of 5 structural analogues of Puromycin that were investigated, only one, 6-dimethyl-amino-purine-3-amino-d-riboside, produced a nephrotic syndrome in rats. The specific structural configuration necessary to induce this syndrome is discussed.

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Influence of Dietary Chlortetracycline on Incidence of Urinary Calculi in Sheep.* (23901)

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The development of urinary calculi in sheep and other animals has been associated with many of the commonly used feedstuffs(1,2), with vitamin deficiences(3,4), with unbalances of minerals, particularly of calcium and magnesium(5,6) and of potassium and phosphate (7,8), and with insufficient water intake and other factors. The exact cause of calculi formation in sheep, however, is unknown and only a few materials are known to affect the course of calculi development. Elam, Ham and Schneider(8) reported the reduction of urinary calculi in high potassium acid phosphate diets by the use of 10% NaCl in the diet. Loeschke and Elvehjem(9) noted that the addition of 1 g of NH_4Cl daily to the diets of mink reduced the incidence of calculi.

In an experiment designed to study the effect of chlortetracycline on the utilization by lambs of nonprotein nitrogen (urea) and in diets in different physical states (meal or pelleted), a varying incidence of urinary calculi was noted. This variation in the incidence of calculi with the dietary treatment and its possible implications are the subject of this report.

Procedure. Two hundred seventy-six fine wool wether feeder lambs (averaging 55 lb) were divided by weight into 7 lots of 39-40 lambs per lot. They were self-fed a total mixed diet. The general experimental outline is indicated with results in Table I. Basic constituents of experimental diets were ground milo, molasses, ground alfalfa, and cottonseed hulls. The protein or nitrogen supplements, *i.e.,* cottonseed meal (CSM) or urea, were added at the expense of the milo and cottonseed hulls (footnotes, Table I). The cottonseed meal diets with and without chlortetracycline in the meal or pelleted states were fed to 4 groups of lambs. Pelleted urea diets with and without chlortetracycline were fed to 2 groups of lambs and the low protein diet without antibiotic was fed to the seventh group of lambs. The calculated total digesti-

* This investigation was supported in part by grant from American Cyanamid Co., Pearl River, N. Y. Appreciation is expressed to Dr. F. T. Lynd and C. H. Bridges, Dept. of Veterinary Pathology, A. & M. College of Texas, for assistance in autopsies.

TABLE I. Death Losses from Urinary Calculi and Incidence of Calculi in Surviving Lambs.

Group No.	Nitrogen supplement	Physical state	Chlortetracycline, mg/lb feed	No. lambs	Deaths, calculi	Incidence calculi, surviving lambs, %	Avg calculi, surviving lambs, mg/kidney
I	CSM*	Meal	0	40	7	11	3
II			10	40	1	11	2
III	"	Pelleted	0	39	9	44	246
IV			10	39	4	24	5
V	Urea†	"	0	39	2	26	5
VI			10	39	0	6	1
VII	None‡	"	0	40	3	4	1

* Diet composition: 10% cottonseed meal, 45% ground milo, 10% molasses, 8% ground alfalfa, 27% ground cottonseed hulls.

† Diet composition: 1.1% urea, 52% ground milo, 10% molasses, 8% alfalfa, 29% cottonseed hulls.

‡ Diet composition: 52% ground milo, 10% molasses, 8% alfalfa, 30% cottonseed hulls.

ble nutrient contents were equivalent in all diets. Crude protein (N x 6.25) was 11% in the supplemented diets and 8% in the low protein diet. Average Ca and P contents were 0.40% and 0.27%, respectively, in the cottonseed meal diets and 0.43% and 0.22%, respectively, in the urea and low protein diets. When used, chlortetracycline was added to the feed at a level of 10 mg per lb feed as a crude material (Aurofac) containing the equivalent of 1.8 g chlortetracycline • HCl per lb of crude material. Water and salt were available *ad libitum*. All lambs which died during the experiment were autopsied and the entire urinary tract of each was examined for presence of urinary calculi. Upon slaughter of the remaining lambs at termination of a 98-day experiment, one kidney was removed from each of 146 randomly selected carcasses and the kidney was inspected for presence of urinary calculi.

Results. There were 26 deaths due to urinary calculi during the 98-day trial. Calculi were found variably in kidneys, ureters, and bladder, but complete or partial urethral blockage occurred in every instance. When the urethra was stopped completely, apparent infection occurred at site of blockage and the urethra ruptured allowing urine to flow into the body cavity and resulted in death due to uremia within a few days. In several cases the urethra was not completely blocked but urination was impaired and the animal showed symptoms of extreme discomfort, anorexia, and loss of weight. Some animals living 4-5 weeks died as a result of secondary diseases such as pneumonia or diarrhea, but urinary calculi was definitely diagnosed in each case.

Variation in incidence of deaths caused by calculi with various treatments is shown in Table I. Death losses due to urinary calculi were reduced significantly ($p<0.01$) among lambs receiving chlortetracycline in the diet.

At end of experiment, no visible symptoms of urinary calculi were shown by remaining animals but upon examination of kidneys taken from carcasses of slaughtered surviving lambs, calculi were found in 18% of the kidneys. Accretions in most lambs ranged from about 1 mg to 90 mg, but 4 lambs which received pelleted cottonseed meal diet without chlortetracycline showed extensive accumulation of calculi or hydronephrosis with from 370 mg to 4.19 g of calculi in the kidney. The incidence of calculi in surviving lambs and the extent of calculi development (calculated as average calculi/kidney in all examined surviving lambs) tend to support the conclusion which may be drawn from death losses that chlortetracycline reduces development of urinary calculi.

Death losses were significantly fewer with the urea supplemented and low protein diets than with cottonseed meal supplemented diets ($p<.01$). There were no death losses with the urea plus chlortetracycline supplemented diet. Also of importance is that deaths from urinary calculi within the urea containing diets (Groups V and VI) occurred after 80 days while those on cottonseed meal diets began occurring after 30 days on feed.

A greater quantity of renal calculi was found in lambs receiving pelleted cottonseed meal diet than in lambs receiving the same diet in the meal state. Pelleted feeds thus appeared to be more inducive to renal calculi. The 4 death losses in the group receiving pelleted cottonseed meal diet containing chlortetracycline may be indicative of some decrease in effectiveness of chlortetracycline in a pelleted feed, but an analysis of the chlortetracycline in this diet indicated an activity of only 3.8 mg/lb of feed. Since a 10 mg level was added to the feed, it is conceivable that chlortetracycline may be deactivated in the pelleting process where heat and steam are used. The marked reduction of calculi formation in the kidney suggests that chlortetracycline at even this lower level was effective in reduction of renal calculi formation.

The main mineral components of the calculi observed were Ca, 8%; Mg, 5.3%; P, 15%; and carbonate, 18%. The renal calculi were small white particles which in the more severe cases were sedimented together to form easily crushable stones which ranged to a size of over one-half inch in diameter. The occurrence of 1 to more than 4 g of calculi in the pelvis of the kidney of several lambs and an enlargement of the ureter as the stones moved downward suggested that the formation of calculi occurred principally in the pelvis of the kidney.

Discussion. The reduction of incidence of urinary calculi by dietary inclusion of chlortetracycline may point to a new physiological effect of antibiotics in the ruminant.

As to the mechanism by which chlortetracycline may have affected the course of calculi development, a number of possibilities are evident. A hypothesis may be applicable that the antibiotic tends to reduce minor kidney infections thus reducing potential nuclei for calculi formation. The dietary chlortetracycline may maintain a more healthy state in the kidney and thus more effectively regulate the rate of renal reabsorption and excretion of mineral elements. The antibiotic may also reduce the numbers of bacteria producing urease in the kidney and thereby prevent a calculogenic increase in pH of the urine.

Indirect effects, which may be mediated through the action of rumen or other gastro-intestinal tract bacteria, on the physiology and metabolism of sheep are also possible methods of inhibition of calculi formation. Addition of chlortetracycline to the diets of lambs has been shown to increase the efficiency of feed utilization as well as affecting the incidence of bacterial induced gastro-intestinal disorders such as entero-toxemia and diarrhea coincident with placing lambs on a self-fed ration for fattening purposes(12). The intestinal absorption of Ca in chicks can be influenced by dietary antibiotics(10,11) and a similar effect may have occurred in the sheep with consequent inhibitory effects on the development of calculi. Mineral balance studies or analyses of serum Ca and P, however, have not been carried out under the conditions of the present experiment. The apparent aggravation of the development of urinary calculi by pelleting the feed may be further suggestive of an involvement of the activity of rumen microorganisms in the course of calculi development although the mechanism of such involvement is difficult to visualize at this point.

The lower incidence of urinary calculi in the urea supplemented diets may lead to wider interpretation to the observations that urinary calculi are reduced in mink(9) and other animals by the administration of NH_4Cl. The principal intermediate in the metabolism of urea by rumen microorganisms is $(NH_4)_2CO_3$. However, the low level of death losses and low incidence of renal calculi with the low protein diet (Group VII) would indicate that the urea had little effect upon urinary calculi and that other basic feed components contain the calculogenic factors.

Summary. Two hundred seventy-six wether feeder lambs were divided into 7 lots for 98-day fattening period. The animals were self-fed total mixed ground milo-molasses-alfalfa-cottonseed hull diets calculated to contain the same total digestive nutrients with urea or cottonseed meal as a source of supplementary nitrogen. Twenty-six deaths from urinary calculi occurred. There were significantly fewer death losses in groups of lambs fed diets containing 10 mg chlortetracycline/lb of feed than in lambs receiving control diets. Also,

fewer deaths occurred among lambs fed diets containing urea than among those receiving cottonseed meal as a source of nitrogen. Observations of calculi in the kidney of 146 of the remaining lambs at slaughter revealed a lower incidence and less calculi in those lambs having received chlortetracycline in the diet. The possibility of a physiological effect of dietary chlortetracycline on the reduction of renal calculi formation and subsequent death from urinary calculi is suggested.

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Effect of Intrinsic Factor Concentrates on Vitamin B_{12} Receptor Protein of Tissues.* (23902)

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Reports from this laboratory indicate that intrinsic factor (IF) containing materials stimulate certain serum proteins to combine with Vit. B_{12}(1) and IF concentrates stimulate uptake of radio-active Vit. B_{12} by slices of rat liver or kidney(2). The data presented here demonstrate that IF containing materials stimulate receptor proteins to combine with Vit. B_{12}. These proteins are distributed in all tissues thus far tested. Furthermore, the data offer support to the view that IF stimulates uptake of Vit. B_{12} in slices by first being absorbed into the cell.

Methods and materials. Wistar strain, adult rats grown on stock diet were used. The animals were decapitated and organs to be studied quickly removed and chilled on cracked ice. Homogenates, 10%, were prepared in buffer by a Potter-Elvejhem homogenizer. Incubations, 1 hr, were carried out in standard Warburg apparatus at 37°. The main chamber contained 1 ml of homogenate, intrinsic factor concentrate (IFC)† dissolved in buffer(3) and buffer to make a volume of 2.6 ml. The side arm contained 0.4 ml of cobalt$_{60}$ labeled Vit. B_{12}‡ in buffer which contained 30 mμg of Vit. B_{12} and approximately 0.05 μc of radioactivity. The final volume was 3 ml. The system was aerated 5 min. with 20% O_2, 5% CO_2, and 75% N_2; after allowing a 5 min. period for temperature equilibration, the vitamin was tipped-in from side arm. The experiment was terminated by aspirating contents of each flask into a pipette and transferring to test tube. To each test tube was added 60 mg charcoal, suspended in one ml of buffer. The charcoal quickly adsorbs the free Vit. B_{12} and stops the reaction. All tubes from each experiment were shaken

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† Intrinsic factor concentrate was supplied by Abbott Laboratories, North Chicago, Ill. Its biological potency was established as 5 mg/day, using urinary excretion test as means of assay.

‡ Radioactive vit. B_{12} was generously supplied by Merck and Co.

TABLE I. Effect of IFC on Amount of Vit. B_{12} Combined by Rat Liver Homogenates.

Conditions	CPM/100 mg of liver	
	Control	100 μg IFC added
Complete incubation mixture*	22,600	22,600
Dialyzed,† incubation mixture (DIM)	6,870	14,700
Supernatant from centrifuged DIM‡	2,700	11,350
Sediment from centrifuged DIM‡	3,995	3,489

* Complete incubation mixture contained 1.0 ml 10% liver homogenate, 30 mμg radioactive B_{12} and buffer or IFC as indicated; final vol 3 ml.

† Dialyzed for 17 hr against 100 vol of isotonic KCl at 5°C.

‡ Centrifuged for 10 min. at 25,000 × g.

5 minutes in a mechanical shaker. These tubes were then centrifuged at 25,000 x g for 10 minutes. Clear supernatant fluid was decanted into cuvettes for assay in a center-well scintillation counter. Data are expressed as counts/minute (CPM) corrected for background which was about 600 CPM for our instrument.

Results. It was necessary in preliminary experiments to use dialysis to remove free Vit. B_{12}. In Exp. 2 (Table I) the addition of IFC to the reaction mixture caused a 110% increase in combined Vit. B_{12}. When incubation mixture which contained IFC was cleared of cell nuclei, membranes and mitochrondria by centrifugation, the resulting supernatant fluid contained a 320% increase in combined Vit. B_{12}; whereas the sediment did not show increase. These data demonstrate that IFC stimulates the soluble protein and the microsomes rather than cell nuclei, membranes or mitochondria to combine with Vit. B_{12}. Also, these data support the view that increased uptake of Vit. B_{12}, by tissue slices, reported previously(2), was the result of IFC having been absorbed by the liver slice and thus exerting its stimulatory action intracellularly rather than extracellularly.

It was demonstrated that 75 to 93% of the action of IFC is heat labile, (Exp. 3 and 5, Table II). The disturbing factor, however, in these experiments was the observation that pink cobalamin protein,§ which has no intrinsic factor activity was also potent in stimulating liver homogenates to combine with Vit. B_{12}; this difficulty was resolved in subsequent experiments. The experiments thus far demonstrate that stimulatory action of IFC is associated with soluble proteins of cell or microsomes. This being the case it was no longer necessary to use the cumbersome and tedious dialysis procedure. Thus charcoal was used to remove free Vit. B_{12} and this served to accentuate the stimulatory action of IFC 10-fold, or more, Exp. 5, (Table II). Data in Table III demonstrate that stimulatory action of IFC is operative in both the microsome fraction of liver homogenates and in the soluble portion, to approximately an equal extent.

An extract of liver was prepared by centrifuging a 10% homogenate at 25,000 x g for 15 minutes at 5°, discarding the residue. This extract could be stimulated by IFC to combine Vit. B_{12} (Table IV). The extract was made 30% saturated with $(NH_4)_2SO_4$, and resulting precipitate collected, dissolved in

TABLE II. Effect of Heated IFC, Cobalamin Protein(8) and IFC on Amount of Vit. B_{12} Combined by Rat Liver Homogenates.

Conditions	CPM/100 mg of liver		
	Control	100 μg IFC added	Other additions
Complete incubation mixture*	22,600	22,600	
Dialyzed incubation mixture (DIM)†	8,600	14,510	
DIM	8,600		9,990‡
DIM	8,600		14,230§
DIM, Exp. 2 treated with 50 mg charcoal	405	4,300	
DIM, Exp. 3 treated with 50 mg charcoal	405		690‡
DIM, Exp. 4 treated with 50 mg charcoal	405		4,850§

* Complete incubation mixture contained 1.0 ml 10% liver homogenate, 30 mμg radioactive B_{12} and buffer or IFC as indicated; final vol 3 ml.

† Dialyzed for 17 hr against 100 vol of isotonic KCl.

‡ Intrinsic factor concentrate was dissolved in buffer and heated in a boiling water bath for 30 min. prior to use in these exp.

§ Pink cobalamin protein(8) was added, 100 μg per flask.

§ Pink cobalamin protein was supplied by Dr. K. W. Thompson of Organon Laboratories.

TABLE III. Effect of IFC on Amount of Vit. B_{12} Combined by Soluble Proteins and Microsomes from Rat Liver.

Conditions	CPM/100 mg of liver Control	100 μg IFC added
Complete incubation mixture*	19,000	19,000
Dialyzed incubation mixture (DIM)†	5,500	11,950
DIM, microsomes‡ substituted for liver homogenate	4,950	12,714
DIM, soluble proteins§ substituted for liver homogenate	5,260	12,714
DIM, from Exp. 2, treated with 60 mg of charcoal	302	5,797
DIM, from Exp. 4, treated with 60 mg of charcoal	147	6,745

* Incubation mixture contained 1 ml of 10% liver homogenate, 30 mμg radioactive vit. B_{12} and buffer or IFC as indicated; final vol 3 ml.

† Dialyzed for 17 hr against 100 vol of isotonic KCl.

‡ and § Microsomes were prepared from liver homogenate by first removing mitochondria and other large particles by centrifuging for 5 min. at 30,000 × g. The microsome fraction was sedimented by centrifugation for 2 hr at 110,000 × g. The material that resisted centrifugation under these conditions is designated as soluble protein (§) fraction.

water and dialyzed against 100 volumes of isotonic KCl. After dialysis this preparation was not stimulated by IFC to combine with Vit. B_{12}, (Exp. 4, Table IV). Additional $(NH_4)_2SO_4$ was added to make the extract up to 66% saturation. The precipitate which resulted was dissolved in water, dialyzed as above, and contained about 90% of the activity of original dialyzed extract Exp. 5, Table IV. Of considerable interest is the observation that pink cobalamin protein was only 6% as active as IFC, which is in contrast to the observation that it was equally as active as IFC when whole homogenate was used in the incubation mixture. The protein that was soluble in 66% $(NH_4)_2SO_4$ was dialyzed as before and was inactive in this system, (Exp. 7, Table IV).

Data presented in Fig. 1 show effect of concentration of IFC on stimulation of a liver homogenate to combine with Vit. B_{12}. The response is proportional to concentration of IFC added and this response is linear between 10 and 50 μg. No explanation can be offered, at the present time, for the departure from linearity observed when small concentrations of IFC are added. We have not observed this phenomenon when an extract of liver was first purified with $(NH_4)_2SO_4$. Fig. 2 depicts results obtained when neutralized, lyophilized, normal gastric juice was reconstituted and tested with a 10% rat liver homogenate. Under conditions of these experiments gastric juice alone did not bind significant quantities of Vit. B_{12} although it did

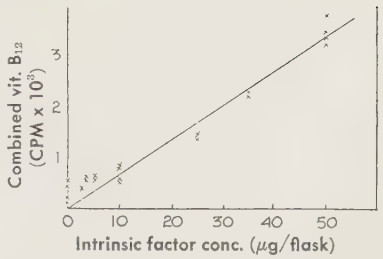


FIG. 1. Combined vit. B_{12} is plotted against the amount of IFC added to each flask. Each flask contained 1.0 ml 10% liver homogenate, 0.4 ml CO^{60}-vit. B_{12}, and 1.6 ml buffer or an appropriate amount of IFC dissolved in buffer.

TABLE IV. Effect of $(NH_4)_2SO_4$ Fractionation of Vit. B_{12} Receptor Proteins from Rat Liver on the Ability of These Fractions to Combine with Vit. B_{12} in Presence or Absence of IFC.

Conditions	CPM/100 mg of liver Control	100 μg IFC added
Complete incubation mixture*	22,800	22,800
Incubation mixture treated with 60 mg of charcoal after incubation (CIM)	100	5,130
CIM, enzyme preparation substituted which had been dialyzed† prior to incubation	100	2,612
CIM, enzyme preparation dialyzed after precipitating with 30% $(NH_4)_2SO_4$ saturation	50	50
CIM, enzyme preparation dialyzed after precipitation between 30-66% $(NH_4)_2SO_4$ saturation	80	2,360
CIM, same as preceding exp. except 100 μg of pink cobalamin protein was substituted for IFC	80	268
CIM, enzyme preparation soluble in 66% $(NH_4)_2SO_4$ and dialyzed†	63	71

* Incubation mixture contained 1 ml of enzyme preparation, 30 mμg of radioactive vit. B_{12} and buffer or IFC as indicated; final vol 3 ml. The enzyme preparation was a supernatant fluid fraction prepared by centrifuging a 10% liver homogenate for 15 min. at 25,000 × g.

† Enzyme preparation was dialyzed against 100 vol of isotonic KCl.

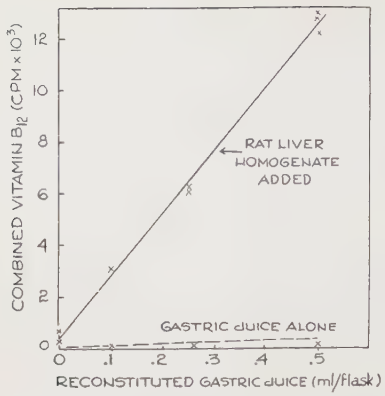


FIG. 2. Combined vit. B_{12} is plotted against the amount of reconstituted gastric juice added to each flask. Each flask contained 1.0 ml liver homogenate, 0.4 ml CO^{60}-vit. B_{12}, an appropriate amount of reconstituted gastric juice, and an additional amount of buffer to make a final vol of 3 ml.

stimulate proteins of liver homogenates to combine Vit. B_{12}.

It was of interest to compare serum proteins and liver homogenates as to their ability to combine with Vit. B_{12} both with and without stimulation of IFC (Fig. 3). It appears that IFC stimulates serum and liver homogenates to combine with Vit. B_{12} to the same extent. One striking difference was observed when one compares the amount of combined Vit. B_{12} without added IFC. About 5 times more Vit. B_{12} was combined by serum than by liver homogenates.

Discussion. Data presented indicate that all of the tissues of the rat, including duodenum, contain receptor proteins which combine with Vit. B_{12} when stimulated by intrinsic factor containing materials. The data reveal that Vit. B_{12} receptor proteins are found in the microsomes and also in the soluble protein fractions of liver homogenates. These B_{12}-receptor proteins are stimulated by reconstituted lyophilized normal human gastric juice and IFC prepared from hog intestinal mucosa. This stimulation is proportional to concentration of added IF containing material. It is of interest that human gastric juice is active in this system whereas it was not active in stimulating uptake of Vit. B_{12} by rat liver slices. These data, together with the reports that human gastric juice(5) hog gastric juice (6) and a commercial hog stomach preparation(7) did not increase the absorption of CO^{60}-B_{12} in the gastrectomized rat, lead us to the tentative conclusion that the active form of intrinsic factor from various species is elaborated in several forms. In order for IF to become available to the host animal the IF concentrate which is secreted or elaborated by the cells of the stomach must be digested in order to present its active site for action on cell surfaces. Should the host animal be unable to do this, the IFC is inactive. We have found that some IF preparations from hog mucosa have varying amounts of activity which stimulate serum receptor proteins to combine with Vit. B_{12}. Some of this activity

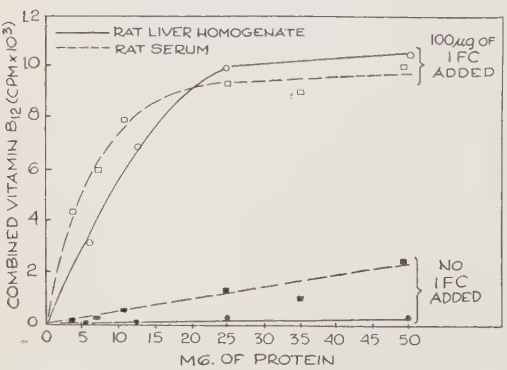


FIG. 3. Combined vit. B_{12} is plotted against mg of protein. Each flask contained 0.4 ml of CO^{60}-vit. B_{12}, an appropriate amount of serum or rat liver homogenate suspended in 2.1 ml of buffer, and 0.5 ml of buffer. 0.5 ml of IFC (200 µg/ml), dissolved in buffer, replaced 0.5 ml of buffer in the experiments indicated.

TABLE V. Comparison of Ability of Homogenates of Various Tissues to Combine with Vit. B_{12} in Presence or Absence of IFC.

Tissue	No. of exps.	CPM/100 mg tissue* Control	100 µg IFC added
None	4		78
Stomach	2	12,148	10,935
Lung	2	463	9,103
Ileum	2	160	8,538
Spleen	4	460	8,479
Skeletal muscle	4	171	7,534
Liver	9	451	7,479
Heart muscle	4	150	6,742
Duodenum	5	356	6,400
Brain	4	75	2,409
Kidney	4	135	1,368

* Incubation mixture contained 1 ml of 10% tissue homogenate, 30 mµg of radioactive vit. B_{12} and buffer or IFC as indicated; final vol 3 ml. After incubation the mixture was treated with 60 mg of charcoal, see text for details.

is not heat labile. However, when these same preparations are treated with crystalline papain all of their activity becomes heat labile.

Although pink cobalamin protein(8) stimulated liver homogenates to combine with Vit. B_{12}, receptor proteins purified by ammonium sulfate fractionation were not stimulated by this curious Vit. B_{12} containing protein. This unexplained phenomenon supports the view that stimulation of purified receptor protein by IFC is a function of its intrinsic factor content. More extensive studies are needed to clarify this point.

A comparison of receptor proteins from serum and liver (homogenates), provides evidence that intrinsic factor may be absorbed into the blood stream of the rat from the intestine. Although other explanations are not precluded, the most plausible explanation of the data shown in Fig. 3 is that rat serum contains a material that acts like IF in the system under investigation. Also, the data presented extend and support a previously stated hypothesis(9,2) that intrinsic factor is absorbed into the blood stream where it acts not only to conserve the dietary Vit. B_{12} by enhancing its combination with receptor proteins of serum but also facilitates absorption of Vit. B_{12} by peripheral tissues by causing it to combine wtih the receptor proteins of the various tissues.

Summary. Data have been presented which show that intrinsic factor containing materials will stimulate receptor proteins to combine Vit. B_{12} of tissues as well as serum. Further, the receptor proteins of tissues are located intracellularly in the microsome and soluble protein fraction of liver homogenates. The evidence suggests that intrinsic factor aids in absorption of Vit. B_{12} by stimulating the intracellular receptor proteins to combine with Vit. B_{12}.

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Competitive Transfer of Sorbose and Glucose in Placenta of Rabbit. (23903)

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Previous studies on the placental transfer of simple sugars have suggested that an active transport mechanism is involved rather than one of simple diffusion(1-5). A differential permeability of the placenta toward the aldo- and keto-hexoses, respectively, has been noted in all species studied (sheep, guinea pig, rat, rabbit, monkey). In at least one of these (monkey) a competitive inhibition of fructose from mother to fetus by excess of glucose in maternal circulation has been suggested(4).

The purpose of the present study was to investigate the possibility of a competitive inhibition of sorbose transfer by glucose in the placenta of the rabbit at term. The findings indicate a significant depression of the "permeability constant" for sorbose in the presence of excess glucose.

* Student Research Fellows.

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Materials and methods. Rabbits in the last 5 days of pregnancy, *i.e.* from about 25th to 29th day, were used. The experimental procedure has been fully described elsewhere(5) and will be only briefly outlined here. Animals were anesthetized with sodium nembutal and the abdomen opened. Maternal and fetal blood samples were obtained at serial intervals during intravenous infusion of l-sorbose alone or l-sorbose combined with d(+) glucose. A constant speed motor-driven syringe delivering 1.6 ml/minute was used. The jugular vein was cannulated with a polyethylene tube. Since serial blood samples in a single fetus are technically difficult to obtain in the rabbit, whole fetuses were removed at times selected. Two fetuses were removed at a time, each acting as a control for the other. The method gives reproducible results, and its validity has been discussed(5). The type of anesthesia also was shown to be without deleterious effect on permeability of the placenta to the simple sugars. An arbitrary "permeability coefficient," as originally devised by Karvonen and Raïhä(3), was calculated after graphic recording of the maternal and fetal blood sugar levels over the time interval selected. It was calculated as:

$$K = \frac{\text{Rise in fetal blood sugar in mg \%}}{\text{Time in min.} \times \text{avg maternal-fetal gradient in mg \%}}$$

and is thus expressed as the rise in fetal blood sugar level/minute/mg % of gradient from maternal to fetal blood. Total reducing substance was estimated colorimetrically by the method of Somogyi(6). Sorbose was estimated colorimetrically by modified procedure for fructose(7). The latter method gave adequate reproducibility in the presence of large

TABLE I. Permeability Coefficient (K) for Sorbose Alone in the Rabbit Placenta Near Term.

% sorbose infused	Infusion interval in min.	K
10	11–46	.0095
10	13–44	.0099
15	14–40	.0115
15	10–39	.0121
15	16–37	.0116
20	14–41	.0082
40	15–39	.0119

Mean coef. = .0110. Stand. dev. = ± .0017.

TABLE II. Permeability Coefficient (K) for Sorbose in Presence of Excess Glucose in Rabbit Placenta Near Term.

% sorbose infused	Infusion interval in min.	K
15	12–36	.0029
15	15–39	.0044
15	16–41	.0046
15	15–48	.0041

Mean coef. = .0040. Stand. dev. = ± .00076.

excess of glucose. Glucose was observed to give "apparent sorbose" values of 1 to 3% so that corrections were made by use of duplicate blanks. In any event, the direction of the error due to glucose was in favor of increased placental transfer of sorbose rather than the reverse.

Results. Infusion of l-sorbose alone. The permeability coefficient K was first calculated for sorbose alone (Table I). From these figures it is apparent that no significant variation in value of K takes place with increasing concentrations of sorbose in the infusion mixture.

Infusion of d(+) glucose and l-sorbose. An attempt was made to saturate the placental transfer mechanism by preliminary infusion of 50% aqueous solution of glucose. By this means a maternal blood glucose level of about 2000 mg % was achieved. The placental transfer of sorbose was then determined, as described above, by a continued infusion of 15% sorbose combined with 15% glucose. It was found necessary to add this amount of glucose to the sorbose solution to maintain the maternal glucose at the aforementioned level. The results are shown in Table II. The difference of means for sorbose alone and for sorbose in the presence of glucose is significant ($p =< .001$) and indicates a depression of permeability coefficient for sorbose by glucose.

Discussion. The results indicate that there is a significant inhibition of sorbose transfer from mother to fetus in the presence of an elevated maternal blood glucose. Such an inhibition could reasonably be expected if glucose and sorbose share a common mechanism during their passage across the rabbit placenta. It is possible that a "carrier" mechanism might be involved, as suggested on the basis of kinetic data in sheep by Widdas(1),

although no evidence is available as to its nature. That it is impossible to saturate the placental transport system even by elevating maternal blood glucose to unphysiological levels, often fatal, has been observed(5). Thus, a complete inhibition of sorbose transfer could not be obtained by raising the level of maternal glucose. It is possible, of course, that more than one mechanism may be involved. The elucidation of the character of the placental mechanism for transport of sugar remains to be accomplished.

Summary. Sorbose alone and with glucose have been infused intravenously into pregnant rabbits near term, and permeability coefficients for sorbose under these 2 conditions have been calculated. It has been shown that there is a significant depression of permeability coefficient for sorbose when the maternal blood is overloaded with glucose. The results have been interpreted as indicating a competitive inhibition by glucose of sorbose transfer across the placenta.

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Role of Common Ions in Production of Increased Oxygen Consumption by Ouabain.* (23904)

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Since Ringer published his classical papers (1) it has become increasingly apparent that the common ions potassium and calcium alter the heart beat. More recent work(2-5) indicates that the toxic effects of digitalis may be altered by potassium and calcium, and the reports of Holland, Greig, and Dunn(6) and of Vick and Kahn(7) show that digitalis may enhance loss of potassium from the heart. Because of the importance of these common ions to the heart beat and to some actions of digitalis, it was thought desirable to investigate their relation to another effect of digitalis, namely increased oxygen consumption.

Methods. Hearts from embryos of chicken eggs incubated 5 days were dissected into Locke solution of the following composition: NaCl 9.00 g; KCl 0.42 g; $CaCl_2 \cdot 2H_2O$ 0.32 g; $NaHCO_3$ 0.20 g; dextrose 1 g; the solution was adjusted to pH 7.2-7.4. The hearts were then placed in a cartesian diver and experiments were performed as previously described (8,9). After a 10-minute period the same solution (for control experiments) or ouabain was added and observations on beat and measurement of oxygen consumption continued for 30 minutes. Similar experiments were carried out using each of following solutions: Locke without potassium; Locke with 4 × potassium; Locke without calcium; Locke with 4 × calcium (sodium bicarbonate was omitted). The hearts were kept in these solutions for about 30 minutes before observations and measurements were started. The results of each experiment were graphed, and a master graph for each group of experiments was constructed by averaging and graphing oxygen consumption at each even-numbered minute. Control experiments(8), recently supplemented by others, indicate that oxygen consumption during the entire 40-minute period is linear; therefore a curve of control oxygen consumption of each experiment was obtained by extending the curve of oxygen consumption during the 10-minute control period by dotted

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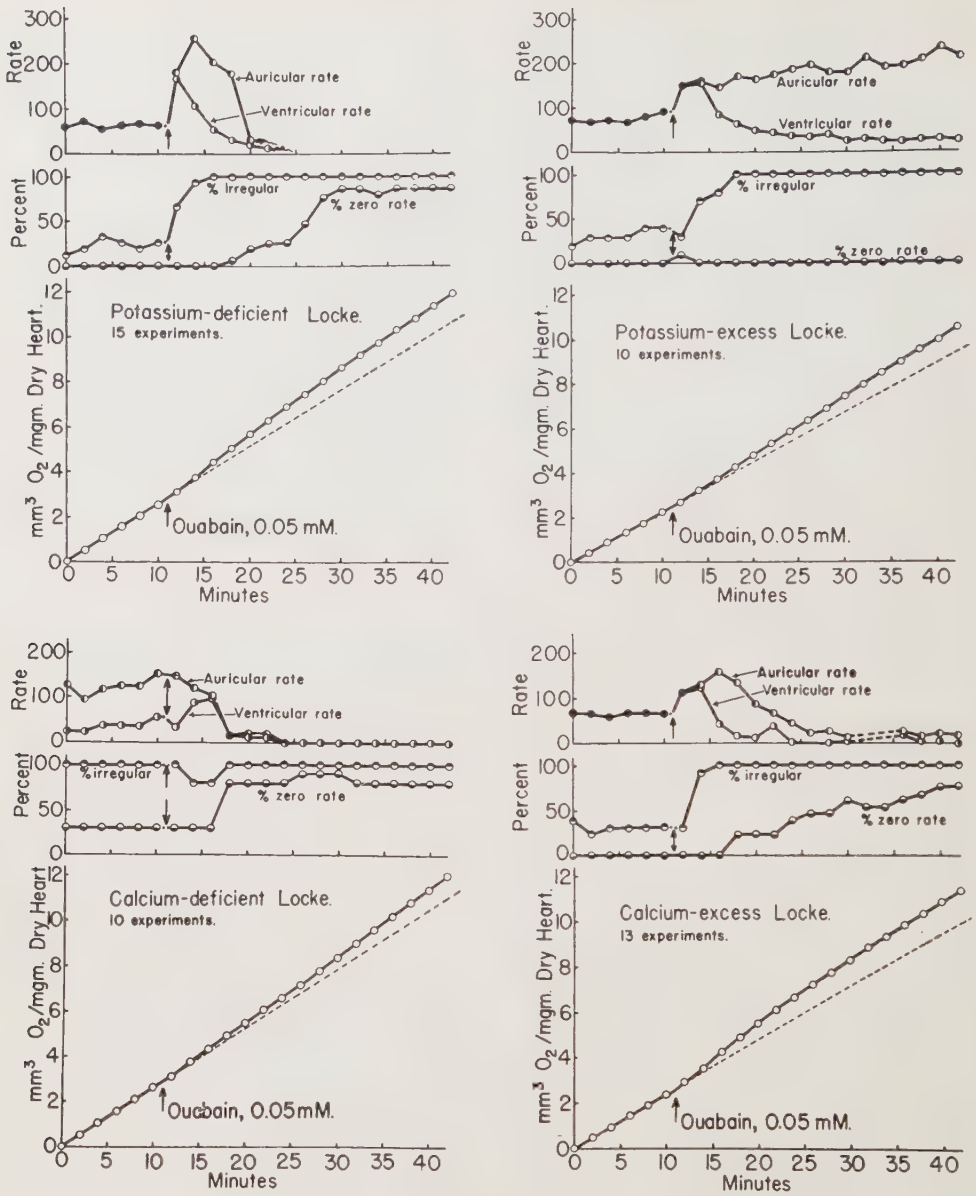


FIG. 1. Effects of ouabain on beat and oxygen consumption of embryonic chick hearts in modified Locke solutions.

line through the following 30-minute period; thus each experiment served as its own control. For statistical purposes, control oxygen consumption was expressed as mm^3 O_2/mg dry heart/hour, and % change in oxygen consumption was computed as experimental consumption/control consumption • 100. Mean and standard deviation were then calculated for each group of experiments. In comparing 2 means a probability of less than 0.01 ($p = <0.01$) was regarded as indicating a significant difference.

Results. Results of these experiments and computations are shown in Table I and Fig. 1. Table I A indicates that the solutions did not alter oxygen consumption. Table I B indicates that ouabain caused a statistically significant increase in oxygen consumption in all

TABLE I.

A. Oxygen consumption in various solutions

Solution	No. of exps.	Oxygen consumption, mm^3 O_2/mg dry heart/hr
Locke	61	15.93 ± 3.704
K-deficient Locke	19	15.20 ± 4.518
K-excess "	15	13.32 ± 2.710
Ca-deficient "	23	16.67 ± 3.883
Ca-excess "	13	14.19 ± 4.867

B. Change in oxygen consumption

Solution	Drug added	No. of exps.	Exp., mm^3 O_2/mg dry heart/30 min. / Cont., mm^3 O_2/mg dry heart/30 min. .100
Locke	Locke (control)	49	100.32 ± 9.680 %
"	Ouabain, 0.05 mM	23	117.50 ± 19.750 %*
K-deficient Locke	*Idem*	15	116.43 ± 20.355 %*
K-excess "	"	10	115.57 ± 13.545 %*
Ca-deficient "	"	10	112.91 ± 14.951 %*
Ca-excess "	"	13	118.72 ± 30.819 %*

* $p = <0.01$.

± refers to stand. dev. in all cases.

solutions. Fig. 1 shows that increased oxygen consumption produced by ouabain in each solution is similar to that previously described in Locke, Ringer, etc., solutions(8). Fig. 1 shows that in calcium-deficient Locke, the beat before and after ouabain is so distorted that effects of the drug are difficult to evaluate. However, the Fig. also shows that heart rate in other solutions is lower but the incidence of irregularities is the same as in Locke, Ringer, etc. solutions(8). Also the effects of ouabain on beat are basically the same in all solutions: initial increase in rate, then increase in irregularities, and eventually increase in incidence of standstill. And finally, these effects are intensified in potassium-deficient and calcium-excess Locke and reduced in potassium excess Locke solution.

Discussion. These observations on the beat verify results published previously that toxic effects of ouabain are intensified by potassium deficiency and calcium excess, and are reduced by potassium excess.

Despite differences in toxic effects on beat of hearts in solutions containing abnormal quantities of potassium and calcium, ouabain increases oxygen consumption identically in all solutions. Therefore it is probable, as postulated earlier(8), that the action of ouabain in increasing oxygen consumption is independent of its action on beat of heart.

Summary and conclusions. Ouabain causes increased oxygen consumption of embryonic chick hearts in Locke solution and in Locke solutions whose potassium or calcium contents had been radically decreased or increased. The effects of ouabain on the beat are exaggerated in potassium-deficient and calcium-excess solutions and reduced in potassium-excess solutions. Increased oxygen consumption produced by ouabain is not dependent on changes in beat of the heart.

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Influence of Antihistamines on Mast Cell Disruption Following X-Irradiation.* (23905)

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It has been shown that tissue mast cells contain histamine(1), and that they undergo widespread disruption in animals exposed to x-irradiation(2-3). More recently, the claim has been made(4) that antihistamine, pyrilamine maleate, prevents destruction of mast cells seen in the x-irradiated hamster. The present paper is concerned with experiments in which antihistamine was administered to the irradiated rat and irradiated hamster. The results suggest that the protective effect is species specific and confined to the region of application of the antihistamine.

Methods. At various times after treatment with x-rays and antihistamines, male, 200-g Sprague-Dawley rats and 100-g Syrian hamsters were sacrificed with ether anesthesia. Samples of mesentery, skin and cheek pouch were removed, prepared as whole mounts(2), stained with toluidine blue(2) and examined under microscope at magnifications up to 1000 ×. To evaluate damage from irradiation and protection by antihistamine treatment, separate counts were made of typical mast cells and of all other cells containing metachromatic inclusions. Of the latter a few are abnormal mast cells(2), while most are fibroblasts and macrophages which contain metachromatic material phagocytized from disrupted mast cells. Thus this count is an indication of amount of damage and disruption of mast cells. Irradiations consisted of single, whole-body exposures to x-rays (250 KV, 15 ma, 0.5 mm Cu and 3.0 mm Bakelite filters, 26.7 cm target distance, 1.5 mm Cu half-value layer, and 215-225 r/min). The antihistamines, pyrilamine maleate† (Neo-Antergan, Merck) and promethazine hydrochloride (Phenergan, Wyeth) were dissolved in Tyrode's solution and administered intraperitoneally each day beginning on day of irradiation.

Results. Both in rat and hamster, x-irradiation was followed by significant damage and disruption of mast cells, as indicated by increases in number of abnormal mast cells and of macrophages and fibroblasts containing metachromatic material (Table I). Administration of pyrilamine maleate after exposure of hamster to 900 r greatly reduced the signs of mast cell damage in the mesentery but not in the cheek pouch or skin (Table I). In similar experiments, not tabulated here, similar results attended treatment with pyrilamine maleate after exposure of hamster to 600 r or 1200 r. In the irradiated rat pyrilamine maleate was without influence on mast cell damage (Table I).

Antihistamine, promethazine hydrochloride, in dosage of 5.0 mg/kg/day was without influence on disruption of mast cells in the mesentery, skin and cheek pouch of hamsters exposed to 900 r or 1200 r. In these experiments groups of 3 animals were sacrificed on days 1, 2, 3, 5 and 7 after irradiation and/or treatment with promethazine hydrochloride. Administration of 25 mg/kg/day of this compound to irradiated hamsters was attended by greater evidence of mast cell disruption in the mesentery than that caused by x-radiation alone (Table I). In nonirradiated hamsters this dosage of promethazine hydrochloride over 6 days caused complete disappearance of mesenteric mast cells (Table I) but had no effect on mast cells of the cheek pouch.

Discussion. The present evidence for damage to mast cells as a result of x-irradiation agrees with our previous findings(2,3,5). The present data clearly show that in the x-irradiated hamster, pyrilamine maleate prevents most or all damage to mast cells of the mesentery but does not affect such damage in skin and cheek pouch. The experiments do not yield information either on the mechanism of protection or on the basis of restriction of protection to the region of application of the anti-

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† Supplied through the kindness of Dr. G. E. Brightenback, Merck and Co., Inc., Rahway, N. J.

TABLE I. Influence of Antihistamines on Damage to Mast Cells in the X-irradiated Animal.

Treatment	Days after irrad.	Cell counts*: Mesentery: Typical mast cells	Mesentery: Phagocytes†	Cheek pouch: Typical mast cells	Cheek pouch: Phagocytes	Skin: Typical mast cells	Skin: Phagocytes
I. Hamster—Pyrilamine maleate							
900 r	1	225	20	412	1	652	0
	2	206	85	748	3	726	3
	3	186	63	452	6	463	16
	5	143	94	643	33	408	8
	7	100	133	349	52	372	47
900 r + pyrilamine maleate, 5.0 mg/kg/day intraper.	1	253	2	138	2	539	2
	2	237	2	524	4	547	3
	3	298	5	373	16	553	27
	5	168	39	345	78	145	52
	7	236	20	264	113	421	16
Pyrilamine maleate, 5.0 mg /kg/day intraper.		257‡	3	407	1	512	0
Untreated controls		245	2	603	0	603	0
II. Rat—Pyrilamine maleate							
800 r	2	128	45			374	173
	5	72	29			251	71
	7	102	7			264	3
800 r + pyrilamine maleate, 5.0 mg/kg/day intraper.	1	171	70			476	109
	2	194	34			328	73
	3	156	27			331	215
	5	154	9			251	29
	6	81	18			283	9
	7	114	7			327	0
Pyrilamine maleate, 5.0 mg /kg/day intraper.		192‡	1			583	1
Untreated controls		201	2			623	4
III. Hamster—Promethazine hydrochloride							
900 r + promethazine hydrochloride, 25 mg/kg/day intraper.	1	94	335	376	7		
	2	71	61	365	6		
	3	158	128	431	38		
	5	0	0	550	88		
	7	0	0	265	79		
Promethazine hydrochloride, 25 mg/kg/day intraper.	1	182	0	639	2		
	2	70	159	262	3		
	3	159	0	1201	12		
	5	0	0	499	0		
	6	0	0	365	0		
	7	15	18	661	5		

* Cells per 2.028 mm². Each value represents avg of 3-6 animals.

† Comprised of fibroblasts and macrophages containing metachromatic material and a few abnormal mast cells.

‡ Avg of 18 animals—3 animals sacrificed on each of days 1, 2, 3, 5, 7, and 9. Values are lumped since there were no significant differences from day to day.

histamine. It is of interest to note, however, that pyrilamine maleate has been reported (1) to prevent the disruption of mast cells caused by injection of stilbamidine, d-tubocurarine and agar serotoxin.

Our finding that pyrilamine maleate failed to reduce disruption of mast cells in the irradiated rat demonstrates a distinct species specificity for the effectiveness of this compound. The absence of protection in hamsters treated with promethazine hydrochloride shows that not all antihistamines are effective.

The mechanism of damage to mast cells by higher dosages of promethazine hydrochloride is not shown by present data. It should be noted, however, that this destructive effect was restricted to the region of application and did not involve mast cells in distant tissues.

Summary. X-irradiation was followed by evidence of marked damage to mast cells in mesentery, skin and cheek pouch of rat and hamster. Daily intraperitoneal injection of pyrilamine maleate largely prevented damage to mast cells in the mesentery but not to those in skin and cheek pouch of the irradiated hamster. Pyrilamine maleate offered no protection against damage to mast cells in tissues of the irradiated rat. Intraperitoneal injections of promethazine hydrochloride in low dosage failed to protect mast cells against radiation damage in any tissue of the hamster. In high dosages this compound destroyed mast cells of mesentery of the nonirradiated hamster, but did not affect those of skin and cheek pouch.

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Effect of Tetracyclines on Formation of Amines by Bacteria.* (23906)

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Many intestinal bacteria are known to produce amines from amino acids(1). Melnykowicz and Johansson(2) found that rats fed small amounts of tetracyclines contained in their gastrointestinal tract less amines than control animals. They found that tetracyclines interfered with formation of some amines by the intestinal flora.

The effect of tetracyclines on formation of amines by single strains of bacteria isolated from human feces was therefore studied. Formation of isoamyl amine and isobutyl amine by *Proteus vulgaris* and of tyramine and phenyethyl amine by *Streptococcus fecalis* is the subject of the present communication.

Methods. Test organism and antibacterial assay. Str. fecalis and *Proteus vulgaris,* isolated from feces of patients suffering from gastro-enteritis were used. Sensitivity towards antibiotics was determined by 2-fold dilutions in nutrient broth (Difco). *Str. fecalis* was inhibited by 2 μg/ml oxytetracycline hydrochloride, tetracycline hydrochloride or chlortetracycline hydrochloride after 24 hours of incubation. *Proteus vulgaris* was inhibited by 50 μg/ml tetracycline hydrochloride or by 20 μg/ml chlortetracycline hydrochloride after 24 hours of incubation. *Culture media.* For amine production a casein hydrolysate medium of the following composition was used:

Casein hydrolysate vit. free (NBC) 10%	3	ml
Yeast extract (Difco)	.5	g
Glucose	.1	g
Sodium chloride	.5	g
Tryptophan	.002	g
Salt solution	1	ml
Distilled water to	100	ml

The salt solution was composed of: $MgSO_4 \cdot 7H_2O$ – 22.5 g, $CuSO_4 \cdot 5H_2O$ – 0.5 g, $ZnSO_4 \cdot 7H_2O$ – 0.4 g, $MnCl_2 \cdot 4H_2O$ – 0.15 g in distilled water to 500 ml pH adjusted to 5.5 and the medium autoclaved 20 minutes. For manometric experiments the strains were grown on brain heart infusion medium (Difco) with 1.5% agar. After incubation at 37°C for 14-18 hours the cells were harvested, washed 3 times with 0.85% sodium chloride solution and kept at –5°C until used. *Analytical methods. Determination of volatile amines by DNFB method.* Volatile amines were quantitatively determined by colorimetric method based on dinitro fluorobenzene (DNFB) reaction(3): 1 ml of a sample was alkanized by addition of 2 ml 40% potassium hydroxide solution and steam distilled

* This investigation was supported by grant from Hadassah Medical Organization Research Fund.

TABLE I. Effect of Tetracyclines on Growth of *Proteus vulgaris*.

	Time (hr)				
	0	48	72	96	144
Control (no antibiotics)	5×10^{4}*	7×10^{7}	4×10^{7}	1.5×10^{7}	7×10^{6}
Chlortetracycline, 30 μg/ml	"	6 "	4 "	1 "	4 "
Tetracycline, 100 μg/ml	"	8 "	9 "	5 "	5×10^{7}

* No. of viable cells/ml of casein hydrolysate medium.

in a Markham still. The distillate was collected in a tube containing 0.5 ml of saturated sodium tetraborate solution. This was then treated with 0.25 ml of DNFB solution (0.65 ml of DNFB in 50 ml acetone), heated 10 minutes at 60°C and 0.5 ml of 5 N HCl added (4). After cooling to room temperature the yellow color was extracted with ethyl acetate and estimated in a Klett-Summerson photoelectric colorimeter at 420 mμ. *Paper chromatography.* Measured quantities of solution to be tested were applied to Whatman No. 1 filter paper. Butanol-acetic acid-water (2:1:1) was the solvent and the paper was sprayed with 0.2% ninhydrin in butanol. Quantitative determinations of the material on each spot were made(5). *Determination of amines by ion exchange method.* Amines were separated from amino acids by Amberlite IRC 50(6). The amines were eluted from resin by addition of 1 N H_2SO_4 and the eluate tested by paper chromatography or the DNFB method. *Manometric experiments.* The conventional Warburg method(7) was used for manometric determination of CO_2. Warburg flasks contained 50 mg freeze dried cells or cell-free preparation, 1 ml phosphate buffer pH 5.8 and 0.5 ml substrate. Total volume in each flask was 3.2 ml and the atmosphere was hydrogen to obtain anaerobic conditions. Freeze dried cells were prepared by drying over phosphorus pentoxide. Cell free preparations were obtained by shaking suspensions in a Mickle tissue disintegrator for 1 hour, and centrifuging at 11,000 rpm for 10 minutes.

Results. Effect of tetracyclines on formation of volatile amines by *Proteus vulgaris. Proteus vulgaris* was grown in casein hydrolysate medium in the presence of 30 μg/ml of chlortetracycline or 100 μg/ml of tetracycline. Growth appeared after 30 hours of incubation only. Samples were taken for the count of viable cells and for determination of volatile amines at different time intervals. After 48 and 72 hours incubation there was no significant difference in number of viable cells in the medium with or without chlortetracycline (Table I). Moreover, in the presence of tetracycline, the number of surviving bacteria after 72 and 144 hours was higher than in the control.

In the presence of antibiotics, formation of volatile amines was markedly depressed (Fig. 1). After 6 days, about a quarter of the control amount of volatile amines was formed in the presence of 30 μg/ml chlortetracycline and about a half in the presence of 100 μg/ml tetracycline. Quantitative paper chromatography examination showed that both volatile amines produced, isoamyl amine and isobutyl amine, were affected in the same degree.

Effect of tetracyclines on formation of aro-

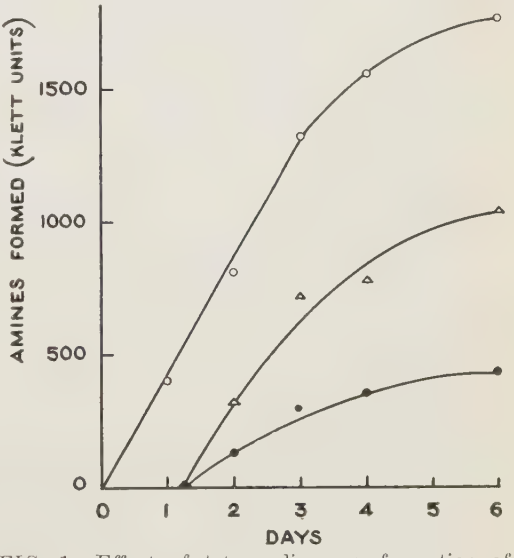


FIG. 1. Effect of tetracyclines on formation of volatile amines by *Proteus vulgaris*. Bacteria grown in casein hydrolysate medium —○—; casein hydrolysate with 30 μg/ml chlortetracycline —●—; or casein hydrolysate with 100 μg/ml tetracycline —△—. Concentrations of amines expressed in Klett units.

TABLE II. Effect of Tetracycline on Decarboxylation of Phenylalanine and Tyrosine by Freeze Dried Cells of *Streptococcus fecalis.*

	Tetracycline, μg/ml				
	1700	170	17	1.7	0
Phenylalanine	0*	17	60	70	77
Tyrosine	39	196	221	215	224

* μl CO_2 evolved/hr.

matic amines by *Streptococcus fecalis.* Tetracyclines in subinhibitory concentrations did not affect formation of aromatic amines by *Str. fecalis* grown on casein hydrolysate medium. High concentrations of tetracyclines, however, inhibited formation of these amines by dried cells or cell free preparations. Manometric determinations showed that 1700 μg/ml tetracycline depressed decarboxylation of tyrosine and 170 μg/ml inhibited decarboxylation of phenylalanine (Table II). Chlortetracycline and oxytetracycline in these concentrations exhibited a similar effect. These findings were confirmed chromatographically.

Discussion. Metchnikoff(8) assumed that bacterial action on proteins in the gut produced harmful amines "ptomaines" which led to enterotoxemia. Evidence is now growing in favor of this view(9). Growth promoting effect of subinhibitory amounts of antibiotics given to young animals might be due to depression of bacterial amine formation(10). The effect of subinhibitory concentrations of tetracyclines on formation of volatile amines by *Proteus vulgaris* might be of clinical interest, even though the effect of isoamyl amine and isobutyl amine on the host has not yet been elucidated. The enzymes responsible for formation of isoamyl amine and isobutyl amine by *Proteus vulgaris* are adaptive(11) and interference of subinhibitory amounts of tetracyclines with formation of some adaptive enzymes has been described(12).

Inhibition of amines produced by *Str. fecalis* has to be looked at from a different point of view. Concentrations of amines about 500 times greater than that necessary for growth inhibition were required to depress formation of phenylethyl amine and tyramine. These high concentrations of antibiotics interfered not only with decarboxylation of tyrosine but also with its deamination(13). Phear and Ruebner(14) found that subinhibitory amounts of tetracyclines had no effect on formation of amines by *Proteus mirabilis,* and had little effect on their formation by *Str. fecalis.* These authors employed a different technic than that used by us.

Our findings that subinhibitory concentrations of tetracyclines slowed down rate of death of cells of *Proteus vulgaris* are in agreement with similar observations reported for chloromycetin(15).

Summary. Tetracyclines in subinhibitory concentrations depressed formation of isoamyl amine and isobutyl amine by *Proteus vulgaris.* High concentrations of tetracyclines inhibited formation of tyramine and phenylethyl amine by *Str. fecalis.*

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Submergence of Bleeding Coronary Artery in Myocardial Tunnel: Experimental Study.* (23907)

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The operation of coronary endarterectomy for some cases of obliterating arteriosclerotic heart disease has been proposed for human patients. Bailey(1) and his colleagues have reported success with this method in clinical cases. The present report describes a study in which a freely bleeding coronary artery has been submerged in a myocardial tunnel. Such a procedure may augment coronary endarterectomy in those cases in which a direct surgical approach to the coronary arteries is contemplated.

Method. Twenty-five mongrel dogs of both sexes varying in weight from 15 to 40 lb were operated on under nembutal anesthesia. Ventilation was carried out with a positive pressure respirator connected to a tracheal cannula. In each animal the heart was exposed through the fourth left intercostal space. The pericardial sac was opened by an incision parallel to the phrenic nerve. The anterior descending or Circumflex Ramus of left coronary artery was dissected free from surrounding tissue, beginning one cm distal to bifurcation of main artery, and extending some 3 cm along length of the vessel. Endeavors were made to retain as many branches as possible of these primary coronary arteries. In all cases, however, it was necessary to ligate small branches, particularly those to the left auricle. Caution was required in freeing the left anterior descending vessel from its venae comitantes and their tributaries. Further hazards arose when dissecting perforating myocardial branches arising from the posterior walls of the coronary arteries. The coronary artery having been dissected free, a site of implantation was prepared on the anterior surface of left ventricle beneath normal course of the vessel. A tunnel not less than one inch in length was prepared, commencing 2 cm from anatomical origin of coronary artery at the aorta. A small curved mosquito forceps was inserted through scalpel incision in the epicardium and eased through the myocardium along path of the future tunnel. At its point of emergence the forceps was spread widely and the myocardium incised along tunnel with a scalpel. The trough so formed was deepened 1 cm towards the endocardium. Bleeding from the myocardial bed was minimal, rarely being more than a slow ooze. Preparations for the endarterectomy incision were then made. A common elastic soft rubber band was passed about the coronary artery proximally so as to be able to occlude blood flow through the vessel at will. In these operations the vessels have been totally occluded for periods to three minutes with no serious consequences. Following tension upon the elastic band with temporary cessation of flow in the selected coronary vessel, an elliptical excision varying in size from 2-3 mm was made with scalpel and Iris scissors in posterior wall of coronary artery. Through this opening a May Curette or Spoon Curette was then introduced into the coronary artery in both directions. Presuming conclusion of endarterectomy, the bleeding artery was then submerged in the preconstructed trench and the myocardium sutured over it with 0000 silk, care being taken not to occlude the vessel at its entrance into, or its exit from, the trench. Release of the elastic band was followed by oozing of arterial blood from the implanted vessel, but this was controlled by gentle pressure. Patency and continued function of the vessels have been demonstrated at autopsy sacrifice. In a few cases, immediately following the procedure, the main coronary artery was severed distal to the tunnel. Regular pulsatile ejection of coronary arterial blood was observed. In no animal operated on in this study did a

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TABLE I. Analysis of Animals Surviving Submergence Operation.

Artery submerged	No. of dogs	Weeks post-surgery at sacrifice	State of submerged coronary artery	Remarks
		10-22 wk		
Left anterior descending coronary artery	11	3 at 10 wk 5 at 12 " 1 at 19 " 1 at 20 " 1 at 22 "	8 patent 2 completely stenosed 1 partially "	Regeneration of endothelium at point of excision, 1 animal at 22 wk. Patency demonstrated by vinyl plastic inj. mass, 8 animals.
		10-22 wk		
Left circumflex coronary artery	6	2 at 10 wk 2 at 12 " 1 at 20 " 1 at 22 "	6 patent	Regeneration of endothelium at point of excision, 2 animals. Patency demonstrated by vinyl plastic inj. mass, 4 animals.

hematoma form about the tunnel. The final stage in the operation was to close the pericardial sac and the chest wall. The anatomical distribution, patency of coronary arteries, and site of endarterectomy incision were evaluated at 9-22 weeks following surgery.

Results. Of the 25 dogs operated on 17 survived for critical analysis of the submergence technic at autopsy sacrifice. The results are summarized in Table I. Two further animals surviving operation died within the first postoperative week of pulmonary causes. One animal was found at post mortem to have had massive atalectasis and pneumonia. The other animal died of distemper. The remaining 6 dogs died during the course of procedure, and all were mortalities in early experience-gaining stage of the study. No fatality was encountered in the last 15 submergence operations. The cause of death in the initial cases was invariably an irreversible ventricular fibrillation accompanied by some degree of blood loss and incorrect closure of the myocardial tunnel. It was noted that a tendency to early ventricular fibrillation was more frequently found to accompany mobilization of the Left Circumflex Coronary Artery.

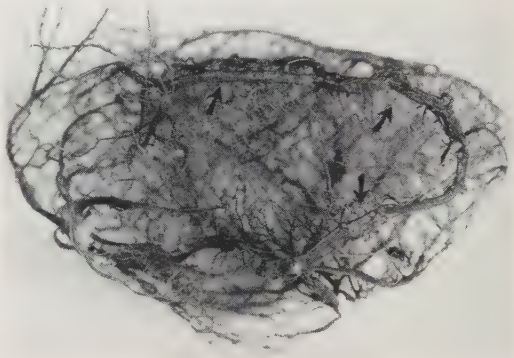


FIG. 2. View from above of circumflex coronary artery (arrows) inj. with vinyl plastic mass 12 wk following submergence operation.

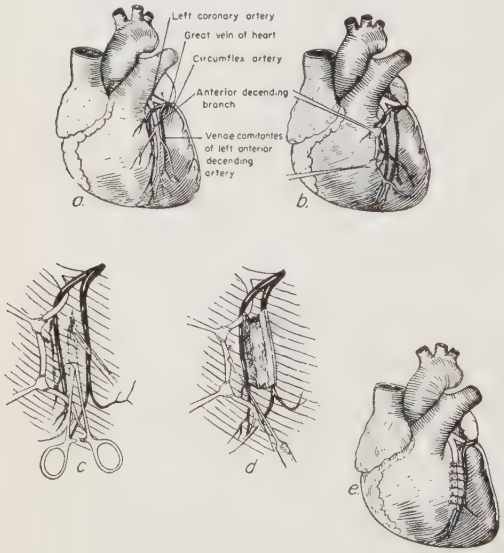


FIG. 1. Submergence procedure for coronary endarterectomy. *(a)* Anatomical structures *in situ*. *(b)* Dissected left anterior descending coronary artery retracted. *(c)* Preparation of submergence tunnel. *(d)* Endarterectomy procedure completed. *(e)* Bleeding coronary artery submerged.

In only 2 surviving animals was a severe complete stenosis of the submerged endarterectomised vessel demonstrated. It seemed probable that such complete obstruction of the coronary arteries was due to an inadequate construction of the tunnel causing angulation of the submerged coronary vessel at the

site of entry to or exit from the submergence tunnel.

Four dogs were sacrificed at 20-22 weeks after operation. There would appear to be no apparent reason why they might not have survived indefinitely, intercurrent disease excepted. In these 4 animals the thoraces were opened under similar conditions to those in the original operation and the submerged vessel was dissected. In 3 cases the vessel was widely patent throughout its entire course, including the segment submerged. In one animal there was narrowing of the vessel with partial obstruction. The sites of excisions in the coronary arteries were identified in these animals as navicular shaped depressions *over which a very thin endothelial membrane had grown* thereby restoring the continuity of the blood vessel wall. The endothelium was "pearly" and clear in other parts of the vessels. In the remaining survivors, sacrifice occurred at a period of 9 to 12 weeks following the submergence operation. The coronary architecture of these animals was investigated utilizing vinyl plastic injection corrosion casts of the coronary arteries(2). No increase in intercoronary anastomoses in the region of the endarterectomy incision and in the area of the myocardial tunnel could be demonstrated when these casts were compared to a standard of anatomy found in fifty normal dogs.

Discussion. The heart muscle is provided with a vasculature more unique and complex than in any other part of the body where arterioles open into a capillary bed which is in turn drained by a system of venules. The architecture of the heart, as in all specialized organs, is peculiar to itself and myocardial muscle presents great differences from skeletal muscle, particularly in its sinusoidal sponge-like character in which true hematomas are seldom, if ever, formed. The present experiments leave no doubt in the vinyl plastic injection casts and the dissection specimens that an incised coronary artery may remain patent with demonstrable blood flow in the majority of animals. An opening left in the coronary artery at time of submergence invariably closes apparently. It would appear unlikely that new collateral branches develop following submergence of a bleeding coronary artery as occurs in some 46% of cases of the Internal Mammary Artery Implantation procedure of Vineberg(3).

A direct surgical attack upon a blocked coronary artery has very much to recommend it as a method of reirrigating an ischemic myocardium, and additionally a point of real interest has been the demonstration of the regeneration of coronary endothelial membrane restoring the continuity of the coronary blood vessel in a normal heart without any late evidence of thrombus or clot formation at the site of excision in the artery.

Summary. 1) An experimental technic developed in dogs is described in which an endarterectomy incision into a normal major coronary artery has been followed by submergence of the freely bleeding coronary vessel into the myocardium. Such a coronary artery was demonstrated to remain patent in 15 of 17 dogs in which the submerged artery was studied at intervals of 9 to 22 weeks after surgery. The procedure has been free from thrombosis that not uncommonly attends insertion of coronary by-pass prostheses or anastomotic procedures upon the coronary arteries. 2) Regeneration of coronary endothelium has been demonstrated at 22 weeks unattended by clot or thrombus formation. It would appear likely that in the operation of coronary endarterectomy upon man the present procedure may simplify the operation and serve as a satisfactory method of controlling the opened vessel in those cases in which pathological change in the coronary artery makes closure of the arteriotomy wound undesirable.

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Production of Anxiety Behavior and Avoidance Conditioning by Stimulation of Bulbar Reticular Formation. (23908)

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Avoidance behavior and conditioning during electrical stimulation of various cortical and subcortical areas have often been reported (1-4). This report presents some observations on behavior of conscious and unrestrained cats during stimulation of the bulbar reticular formation. These observations were made while we were attempting to produce bulbar inhibition of motor activity in intact and unanesthetized cats(5,6).

Procedures. Bipolar electrodes were aseptically and stereotaxically implanted in the bulbar inhibitory areas previously described by Magoun and Rhines(7), following modification of technics used by Segundo *et al.*(8). Stimulus parameters and criteria of placement have previously been described(5,6). Five animals were studied 72-322 hours postoperatively and observations were made on behavioral activity and learned avoidance responses during stimulation of ventro-medial bulbar regions. Learned avoidance responses were induced and measured by 2 different instrumental conditioning technics. The first procedure involved random escape from a round platform in response to bulbar stimulation which was paired with a buzzer sounding 8 seconds previously. The second type of experiment involved a buzzer and light combination stimulus which was followed in 8 seconds by bulbar stimulation. The bulbar stimulation could be avoided when the animal learned to respond by jumping a 6-inch fence barrier. Measures were made of response latencies and percentages of correct responses. Histological confirmation of electrode placements was made following sacrifice.

Results. The usual behavioral response during bulbar stimulation can be described as a series of gradual and consecutive changes from an initially increased but non-directed awareness to apparent uneasiness, apprehension, or anxiety to an ultimate stage of obvious fright. In the last stage, animals which had been extremely friendly prior to stimulation, exhibited such reactions as crouching, scratching at the floor, running behind chairs, hissing and yowling in a highly agitated fashion. Frequently pupillodilatation and piloerection were also noted. Although each animal reacted individually to electrical stimulation, these responses were almost identical with their particular individual reactions to such stressful stimuli as startle noises, squirts of water, etc. However, reactions during bulbar stimulation were not stimulus directed. Similar responses have been described for the monkey by Segundo *et al.*(8) during electrical stimulation of certain cortical and subcortical areas.

Masserman(9) has emphasized the necessity of using conditioning experiments to distinguish between an apparent emotional response and an induced motor response (non-conditionable hypothalamic "rage" reaction of Masserman). To determine whether the responses reported here reflected some negative effect or were complex motor reactions directly induced by stimulation, the animals were conditioned to avoid bulbar stimulation. The block diagram of Fig. 1 indicates the proportion of correct responses in a typical "fence-jumping" experiment. Median response times are indicated by the curve shown in Fig. 1 and appear to approach asymptote at 40 seconds. This long latency corresponds closely to observed time interval required for the gradual succession of behavioral changes described above, in which animals demonstrate a progression from increased awareness to an obviously unpleasant effect after onset of stimulation. In random escape response procedures, cats learned to escape the unconditioned stimulus but did not demonstrate any directional tendencies. This fact suggests that classical sensory components are probably not directly involved in this phenomenon. One control animal stimulated in the dorsolateral medulla manifested jerking and limited turning movements of the head

without exhibiting any of the behavioral phenomena described above.

A photomicrograph of the placement of a typical stimulation electrode is shown in Fig. 2. All electrode placements were similarly limited to ventromedial bulbar reticular areas except for the control preparation described above. Electrode placements generally varied from 5 mm to 12 mm ventral to the floor of the fourth ventricle and in an area bounded 4 mm bilateral to the midline. Antero-posterior dimensions were limited by the cochlear nucleus and the most caudal limits of the medulla. At no time were the classical sensory or motor tracts or cranial nerve nuclei impinged upon.

Conclusions. Behavioral responses, characterized by signs of anxiety, do not solely originate from suprabulbar sites, but also may be produced from ventromedial reticular areas of the medulla. The close correspondence between median response latencies in avoidance conditioning produced during stimulation in this region of the medulla and time intervals elapsing between initial increased arousal and appearance of overt signs of apprehension resulting from the same stimulation, suggests development of a negative "effect" resulting in avoidance behavior rather than direct induction of complex motor responses. Absence of directional tendencies in random escape experiments implies that classical ascending sensory pathways are not directly involved in these effects. Apparently the medullary reticular formation not only participates in respiratory, vasomotor and spinal reflex events but is also involved in certain aspects of conscious behavior associated with emotional signs.

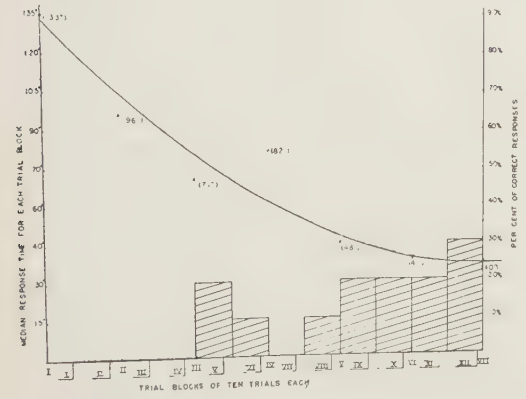


FIG. 1. Correct responses rate and median latency times in a typical "fence-jumping" avoidance conditioning experiment during bulbar stimulation. Each bar represents % correct responses in 10 trials for one animal; 12 hr separated each block of 10 trials. The curve represents change in median *latency* times (in sec.) during the 7 groups of 10-trial blocks for one animal; these are taken as criteria of median *response* times.



FIG. 2. An implanted stimulating electrode site in ventromedial bulbar reticular formation typical of experiments described in text.

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Serum Proteins, Lipoproteins and Glycoproteins. In Muscular Dystrophy of Vit. E-Deficiency.*† (23909)

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Many species of animals develop muscular dystrophy when they are deprived of Vit. E (1) and recover when the vitamin is administered(2,3). The symptoms of this form of nutritional muscular dystrophy resemble in many ways those of patients with muscular dystrophy. Metabolic studies, particularly in rabbits, have revealed a pronounced increase in concentrations of cholesterol and phospholipids in the blood serum and skeletal muscle (4-7). Since the lipids and the bulk of carbohydrates in blood are transported in association with proteins, a closer study of these serum protein complexes seemed to be warranted. It appeared probable that the nature and composition of these lipoproteins and glycoproteins might reflect basic changes in metabolism and thereby give information on the nature of these abnormalities. Previous preliminary investigations(8,9) had shown definite alterations in the moving boundary electrophoretic pattern of blood serum of dystrophic rabbits. In this report the distribution of proteins, lipoproteins and glycoproteins amongst the various electrophoretic fractions of blood sera has been studied in rabbits during development of nutritional muscular dystrophy and during recovery from this disease.

Methods. Muscular dystrophy was produced in young New Zealand rabbits within 3-4 weeks by maintaining animals on Vit. E-deficient diet of Goettsch and Pappenheimer (1), treated with 1% ethereal $FeCl_3$ to eliminate traces of the vitamin. The rabbits weighed 1½ to 2½ lbs when started on this diet. Control rabbits received the same diet and twice a week were given a supplement of 6-7 mg of dl-α-tocopherol (0.25-0.3 ml of 2.5% solution in peanut oil). The same dose of dl-α-tocopherol was administered to effect recovery of dystrophic rabbits. Progress of the disease was followed by determining the ratio of urinary excretion of creatine to that of creatinine(10), which rises to high values in muscular dystrophy. Blood was taken from ear veins after animals had been fasted overnight and the serum separated from the clot by centrifugation. The moving boundary electrophoresis of serum in barbiturate buffer of pH 8.6 and of ionic strength 0.1 was carried out in an Aminco-Stern electrophoresis apparatus at 1°-2°C with a current of 15 mA for 2 hours. Relative concentrations and mobilities were calculated for the ascending pattern. The various electrophoretic fractions were actually separated from each other by means of paper electrophoresis in an LKB apparatus(11). The serum was streaked with Kirk micropipettes across paper strips of 1″ or 1½″ width. Volumes of 10, 30 and 40 λ were applied, depending on whether the electropherograms were to be stained for proteins themselves or for protein-bound lipids or carbohydrates. However, 500 λ were applied on a sheet of 17 cm width when the electrophoretic fractions were to be analyzed for cholesterol and phospholipids. The experiments were done with horizontal strips of Whatman No. 1 filter paper in barbiturate buffer of pH 8.6 and of ionic strength 0.1, at a potential of usually 210 volts and at a current density of 0.1-0.15 mA/cm for about 16 hours at room temperature. The dried strips were stained with Amidoblack 10-B for proteins (11), with Oil Red O for lipoproteins(12), and with periodate-Schiff reagent for glycoproteins(13). The stained bands were scanned with a Photovolt Electronic Densitometer model 525, using narrow band color filters with maximum transmission at 600 mμ, 515 mμ, and 550 mμ respectively. For these measurements the strips stained with Amidoblack 10-B were made translucent by immer-

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† A sample of orosomucoid of known sialic acid content as a standard was kindly supplied by Dr. Richard J. Winzler, Univ. of Ill. College of Medicine.

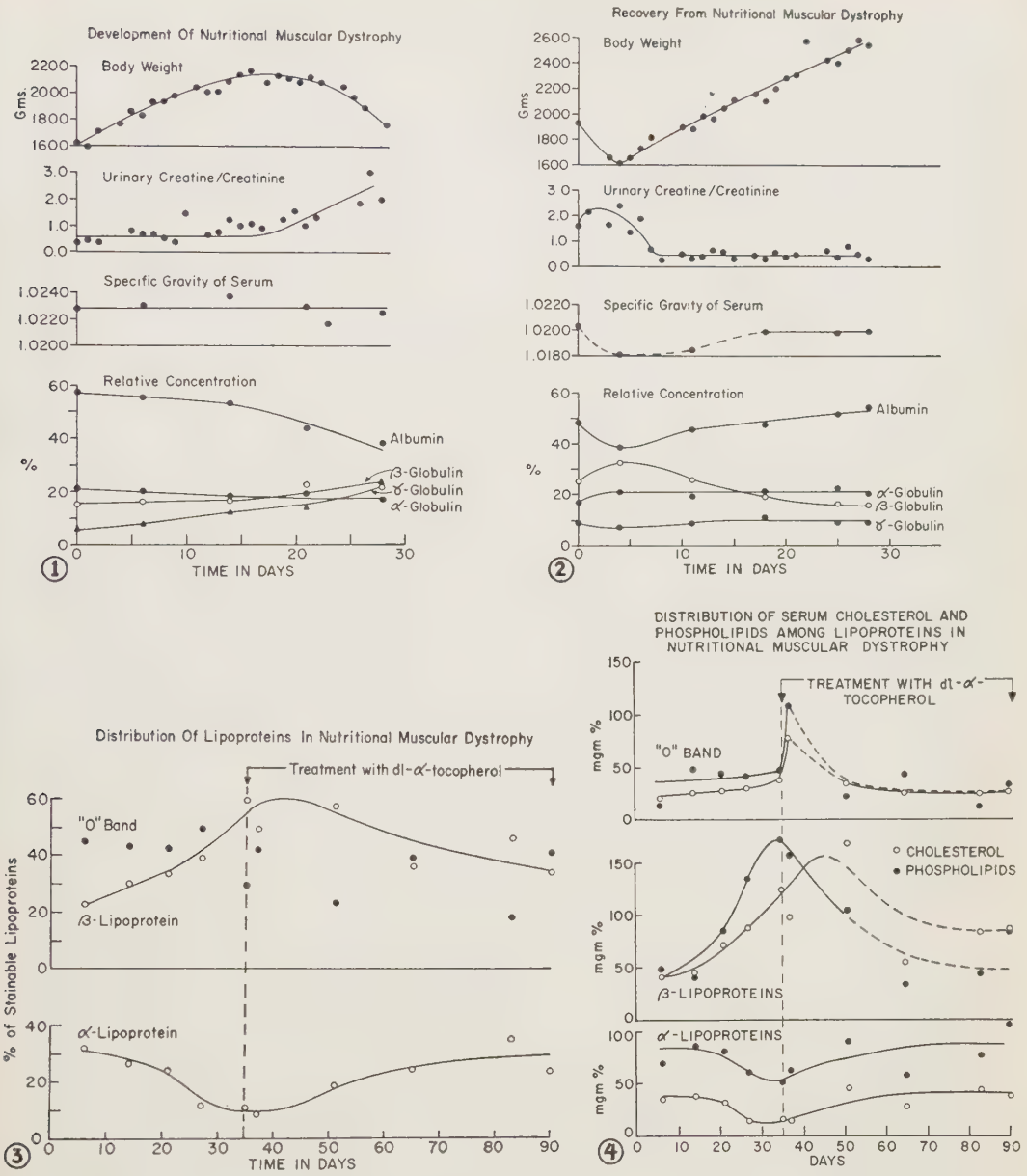


FIG. 1-4.

sion in a mixture of paraffin oil and alpha-monobromonaphthalene(14) or in anisole (15). (Both immersion liquids have a refractive index of 1.51 which is close to that of the paper fibers.) The other stains, however, were measured dry. In the resulting diagrams relative concentrations of electrophoretic fractions were determined with a compensating polar planimeter. Specific gravity of sera was measured in a Lamotte falling drop densitometer(16). The protein nitrogen was determined by the Biuret method(17). The lipoprotein fractions were analyzed for total cholesterol by the Liebermann-Burchard reaction(18) and for phospholipids by the method of Fiske and Subbarow(19). The simultaneous determination of total and of free cholesterol in whole serum was carried out with a

TABLE I. Serum Cholesterol and Glycoproteins in Rabbits during Development of Muscular Dystrophy and during Recovery.

		Cholesterol			Hexoses			
Days	Diet	Free, mg %	Ratio: Free/Total	Esterified, mg %	Total	In seromucoid fraction	Hexosamines	Sialic acid
					mg %			
7	Vit. E-free				75.2	13.0	75.8	65.1
14	*Idem*	28	.22	98	72.4	12.2	73.4	48.7
20	"	55	.29	134	116.5	20.0	91.9	101.0
21	" + tocoph.*							
25	*Idem*	84	.28	213	93.0	10.4	87.6	80.0
28	"	51	.20	202	74.9	10.7	82.4	75.4
32	"	58	.23	196	76.9	11.5	76.9	60.6

* dl-α-tocopherol.

recent method using $FeCl_3$ reagent(20). The glycoproteins were evaluated in terms of the following protein-bound carbohydrates: total hexoses and hexoses in the seromucoid fraction by the orcinol method(21), hexosamines with Ehrlich's reagent(21), and sialic acid with Bial's reagent(22).

Results. Fig. 1 shows the gradual variation of the relative concentrations of serum proteins in moving boundary electrophoretic patterns during development of nutritional muscular dystrophy. It also includes curves for the specific gravity of serum, ratio of urinary creatine:creatinine, and body weight. The latter 2 measurements served to characterize various stages of the disease(2). Decrease of albumin and increase of β-globulin were found in every rabbit investigated and statistically highly significant ($P<.001$). However, changes of the α-globulin fraction which consisted of 3 partly overlapping peaks, and of γ-globulin were not consistent. In contrast to the shift in the distribution of relative concentrations, the mobilities of corresponding serum proteins were alike in dystrophic and normal rabbits. The values for protein nitrogen were also not affected by the progress of the disease. In one rabbit they stayed within the small range of 1.03-1.10 g/100 ml of serum. Fig. 2 shows the effect of treatment of a dystrophic rabbit with dl-α-tocopherol in causing the electrophoretic pattern slowly to return to normal over a period of 4 weeks. Relative concentrations of the various proteins as determined by paper electrophoresis changed with time in a manner generally similar to those in the moving boundary patterns of Fig. 1 and 2.

Distribution of the stainable lipoproteins during development and subsequent cure of muscular dystrophy in a rabbit is presented in Fig. 3. The α-lipoprotein greatly decreased with the progress of the disease and gradually rose again on treatment with dl-α-tocopherol. The β-lipoprotein behaved in a complementary way. Values for the "O" band, which trails the β band and extends towards the origin of the electropherogram(23), scattered considerably, and showed no definite trend.

Fig. 4 shows the amounts of both cholesterol and phospholipids in the α- and β-lipoprotein fractions and in the "O" band. The values have not been corrected for the paper blanks, since the latter fluctuated. The curves varied in a way qualitatively similar to those of the total stainable lipids in Fig. 3. Changes of both lipids in the α-band were more than compensated by their changes in the β-band so that the net result was a considerable rise of their serum levels during the development of the disease and a gradual return to their initial values during period of recovery. The normal values of the ratio of cholesterol to phospholipids for the α- and β-lipoproteins were about 0.5 and 1.0 respectively, which, incidentally, were similar to those reported for human serum(24).

Table I shows that absolute concentrations of both free and esterified cholesterol increased during development of the disease and then decreased slowly during recovery. The ratio of free cholesterol to total cholesterol also increased during progress of the disease, but fell quickly to its original value when the dystrophic rabbit was treated with dl-α-tocopherol.

Variations of protein-bound carbohydrates (hexoses, hexosamines and sialic acid) also are presented in the Table. The concentrations of all of these substances increased, but to a much smaller extent than did those of the lipids when the rabbits became definitely dystrophic, and returned to their normal values within 1-2 weeks after treatment with dl-α-tocopherol had been started.

Staining of protein-bound carbohydrates in the paper electrophoretic fractions did not produce a well-defined pattern. Usually 3 bands were found, at the locations of the α_2-, α_3- and β-globulins. The first 2 bands partly overlapped, which would indicate why occasionally they have been reported as one band in the literature(25). The percentile distribution of these stainable glycoproteins did not seem to vary significantly during development of dystrophy.

Discussion. Changes in the moving boundary electrophoretic pattern during development of nutritional muscular dystrophy were observed earlier than were clinical symptoms and may therefore be used as a more sensitive criterion for onset of the disease. The significant changes in concentrations of albumin and β-globulin represented, to some extent, slow and moderate changes in the proteins themselves as was indicated by paper electrophoretic results. The major changes, however, involved those lipid constituents which are associated with proteins as α- and β-lipoproteins, *i.e.* primarily cholesterol and phospholipids. The large amounts of lipids in the β-fraction probably were mainly responsible for the pronounced increase of β-globulin in the moving boundary electrophoretic pattern during development of the disease. The lipids found in the "O" band are associated only to a small extent with γ-globulin. They consist mainly of substances which have been adsorbed on filter paper, such as chylomicrons (containing neutral fat) and some β-lipoprotein(23).

The observed decrease of α-lipoprotein and corresponding increase of β-lipoprotein are not limited to muscular dystrophy, but have been reported for other diseases in rabbits, particularly atherosclerosis(26,27).

In contrast to the lipids, the protein-bound carbohydrates did not increase nearly as much during progress of the disease and, on treatment, they returned to their normal values more rapidly. The total protein nitrogen did not vary at all. The magnitude of the changes in the lipids and the slow return to normal levels point to the conclusion that disturbances of the lipid metabolism were more severe than were those of the metabolism of proteins and carbohydrates.

Summary. 1. During development of muscular dystrophy of Vit. E-deficiency in rabbits the electrophoretic pattern of serum proteins revealed a decrease of albumin and an increase of β-globulin. These changes were observed earlier than were the usual clinical symptoms and were reversed only gradually during recovery. 2. Concentrations of both cholesterol and phospholipids decreased in α-lipoprotein, but increased greatly in β-lipoprotein so that they rose considerably in whole serum. The magnitude of these changes and the slow return to normal values during recovery indicate severe disturbances of lipid metabolism. 3. In contrast to the lipids, total protein nitrogen did not vary during progress of the disease and the protein-bound carbohydrates increased only moderately and, on treatment, soon returned to their normal levels.

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Effect of Cortisone, Properdin and Reserpine on *Neisseria gonorrhoeae* Endotoxin Activity. (23910)

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In connection with studies on endotoxin derived from *Neisseria gonorrhoeae*(1), we were interested in determining whether certain therapeutic agents would afford a measure of protection against, or enhancement of, toxic properties of endotoxin. We used as therapeutic agents cortisone, reserpine and properdin. Cortisone has been shown by workers to afford some measure of protection against toxic effects of certain bacterial toxins (2). Reserpine and properdin were included since to our knowledge they have not been investigated previously in conjunction with any endotoxin. It was hoped that reserpine and properdin might exert a protective action through interference with possible neurotoxic effect of endotoxin. It has been recently suggested that the properdin system may be a factor involved in natural resistance(3).

Materials and methods. Male CF1 mice weighing 16-18 g, were kept (about 10 animals/cage) in an air-conditioned room and had free access to water and Puritan Mouse Ration. Endotoxin was prepared according to our recent procedure(1). The culture medium, however, has been slightly modified. The nutrient broth has been omitted from the medium and proteose peptone concentration has been increased to 8 g/l. This change resulted in more rapid growth of gonococcus, harvesting being possible in 96 hours. Acetone powder was dissolved in a small volume of 0.05 N sodium hydroxide. The resulting solution was adjusted to pH 7.0-7.4 with 0.25 N acetic acid, diluted to desired concentration with sterile distilled water and administered intraperitoneally. Cortisone (cortone acetate) and reserpine (Serpasil) were diluted with sterile saline to appropriate concentrations and administered intramuscularly. Karber's procedure was used for calculating LD_{50} values and interpreting their statistical meaning(4).

Experiments were conducted as follows: each group of mice was sub-divided into control-challenge and treated-challenge groups. The control-challenge group received only endotoxin, whereas, the treated-challenge group received endotoxin plus one of the 3 agents. Endotoxin controls (LD_{50} 2-3 mg) were run with each experiment since LD_{50} values varied somewhat with each new group of mice. Mice

were observed for a 5-day period.

Results. Cortisone, One Hour After Challenge. In a preliminary experiment a single injection of 1 mg of cortisone 1 hour after challenge, using 5 different endotoxin concentrations with only 6 mice in each group, gave cortisone LD_{50} 3.36 mg/control LD_{50} 2.38 mg, a relative increase in resistance of 41%. This increase was not significant because of small number of mice at each level ($0.05<P<0.1$). To confirm the magnitude of this increase the experiment was repeated with 10 mice for each concentration of endotoxin. The relative increase in resistance of the 10 mice to each of 5 different endotoxin concentrations, based on cortisone LD_{50} 4.29 mg/control LD_{50} of 2.93 mg, was equal to 46%. This 46% increase in resistance to endotoxin was statistically highly significant ($P<0.01$).

Cortisone, One Hour Before Challenge. With 1 single injection of 1 mg of cortisone 1 hr before challenge using 6 mice for each of 5 different concentrations, the relative resistance, based on cortisone LD_{50} 5.33 mg/control LD_{50} 2.38 mg, was equal to 2.24 or 224%. This increase in LD_{50} for cortisone was highly significant even with only 6 mice in each group at each dose ($P<0.01$).

Properdin. The potency of properdin was 9 units/mg. Properdin solutions (reconstituted to isotonicity in sterile distilled water) were directly added to endotoxin solutions just before injection. Five different endotoxin concentrations with 36 to 50 mice/group were used in each of 3 experiments. Listed in Table I are properdin units injected together with endotoxin, LD_{50} values for these mixtures, the ratios of LD_{50} values for these mixtures/LD_{50} of controls. The fairly large dose of 50 units of properdin produced a highly significant increase of resistance of mice to the endotoxin ($P<0.01$). With 25 units and 12.5 units of properdin the resistance was less significant. The 3 groups of experiments were conducted with 3 different lots of endotoxin and mice.

Reserpine. Reserpine was injected 1 hr before challenge. There were 5 different endotoxin concentrations in Exp. 1, and 10 mice in each group. In Exp. 2 there were 6 different endotoxin concentrations and 6 mice/group, except in 3rd and 4th group, in which there were 10 mice/group. In the control there were 5 different endotoxin concentrations and 10 mice/group. It is seen in Table I that 2 μg of reserpine had no effect on endotoxin activity. Five μg of reserpine, however, increased toxicity of endotoxin by 56% which is significant ($P<0.05$). There was no mortality in controls given reserpine, properdin, or cortisone only (not listed in Table I).

TABLE I. Response of Endotoxin to Therapeutic Agents.

Agent	Exp. No.	Schedule of treatment	LD_{50} of mixture, mg	Mixture LD_{50}/control LD_{50}
Cortisone	1	1 mg 60 min. after challenge	3.36	1.41
		Control	2.38	
	2	1 mg 60 min. after challenge	4.29	1.46
		Control	2.93	
	3	1 mg 60 min. before challenge	5.33	2.24
		Control	2.38	
		Units		
Properdin*	1	6.25	3.22	.95
		25	3.80	1.12
		Control	3.40	
	2	12.50	3.36	1.35
		25	4.00	1.61
		Control	2.48	
	3	50	5.10	1.62
		Control	3.14	
		μg		
Reserpine†	1	2	2.93	.97
	2	5	1.68	.56
		Control	3.02	

* Directly added to endotoxin solution.
† Inj. 60 min. before challenge.

Summary. Cortisone significantly protected mice from the lethality of *Neisseria gonorrhoeae* endotoxin. Similar results were obtained with properdin, provided relatively large doses were employed. Reserpine, under certain conditions, significantly enhanced the toxicity of endotoxin.

We are indebted to Dr. Bernard G. Greenberg for the statistical analysis of our results, to Dr. B. E. Sanders of Merck Inst. of Therapeutic Research for generous supply of human properdin, and to Mr. Harold Russell for technical assistance.

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Effect of Alazopeptin (A) on Litter and Fetus of the Rat *in utero*. (23911)

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A. was first isolated and identified from a culture of *streptomyces griseus planus* by DeVoe and collaborators(1). It has the formula of $C_{15}H_2N_7O_6$ with ultra violet and infrared absorption bands characteristic of Diazoketones; it is apparently a peptide consisting of 1 mole of Alpha-alanine and 2 moles of C_6-diazoketoamino acid. A. is very soluble and quite stabile in alkaline media. It inhibited growth of some bacteria as well as Sarcoma 180 in test mice. In previous experiments, 2 other diazoketones(2,3) were extremely toxic to the rat fetus *in utero*. It was, therefore, of interest to examine also the effect of A. on the rat fetus.

Methods. Mature Long-Evans strain rats were used exclusively. The animals were kept on White breeding diet of Long-Evans. Only rats with previous satisfactory breeding performance were employed and mated. The day of onset of gestation was determined by vaginal smear. The morning of massive vaginal sperm findings, females were separated in single cages and so kept until 21st day of gestation to avoid urinary or fecal contact. The day before littering, the animals were sacrificed under ether anesthesia, the uteri removed, placentas, fetuses, sites of implantation noted and recorded. Surviving fetuses were measured, weighed and fixed and later cleared to study malformations. A. was freshly prepared, dissolved in sterile water and injected intraperitoneally without delay. The results of the treatment of three groups of rats are listed in Table II.

TABLE I. Toxicity: Alazopeptin Aa223 Lederle.

	100 mg/kg	150 mg/kg	200 mg/kg
LD_{50}	3/8	6/8	6/8
% wt loss	13	16	17
	.25 mg/kg	.5 mg/kg	1.0 mg/kg
5 LD_{50}	2/6	6/6	6/6
% wt loss	17	15	20

The single LD_{50} for mature Long-Evans rat was 150 mg/kg; the 5 LD_{50} between .25 and .5 mg/kg. All intoxicated rats showed watery diarrhea and fatty livers. Animals dying from effect of compound were dehydrated and lost 20% of initial body wt. Surviving rats recovered quickly within 5 days from single or multiple doses.

* This work performed with grant from U.S.P.H.S. and Population Council.

Results. In *Exp. 1*, .3 mg/kg given on 7th and 8th day of gestation, *i.e.* time of implantation, destroyed all litters completely. 67.5% of the placentas showed remnants of organs in the uteri at sacrifice—usually measuring only 2 to 3 mm in diameter. In *Exp. 2*, two doses of .5 mg/kg, given also on 7th and 8th day of gestation, destroyed 271 of 273 fetuses, with a single fetus surviving in 2 different litters. Both of these fetuses were stunted and would not have survived. Total litter destruction occurred in 26 out of 28 rats, or in 93%. In this series, placental remnants were found in 37% of the resorbed fetuses. In the *third experiment*, .5 mg/kg was given on the 11th and 12th day of gestation, that is at mid term. All litters were completely destroyed and resorbed but, in 81%, surviving placental remnants were found at sacrifice.

The *mother animals*, sacrificed on 21st day of gestation, showed macro- and microscopically no detectable lesions in their internal organs; particularly none in bone marrow, liver or intestinal tract. Practically all animals of the 3 experimental groups showed secretory activity in the mammary glands, corresponding to normal pregnant controls. The two

TABLE II. Alazopeptin; Lederle Aa223.

No. of rats	Day of dose, i.p.	Mother's wt gain		Uteri total implants	Fetuses: Live	Fetuses: Stunted	Fetuses: Resorbed	Surviving placentas	Mothers with total resorbed litters	Litter wt	Fetus: Wt	Fetus: Length
14	7 & 8	+8.3	a	126			129	85	14			
	.3 mg/kg		b	9	0		9					
			c				100	67.5	100			
28	7 & 8	+8.6	a	273	2	2	271	102	26			
	.5 mg/kg		b	9.75			9.7			2.25	2.7	2.8
			c		.6	100*	99.4	37.4	93			
16	11 & 12	+6.75	a	162			162	132	16			
	.5 mg/kg		b	10.1	0		10.1	8.25				
			c				100	81.5	100			

a = Total b = Per mother c = % of total * % of live.

surviving fetuses of Group 2 were stunted and showed on clearing a retarded skeletal development, evidenced by absence of ossifying centers of supraoccipital and nasal bones, as well as metacarpal diaphysis 2, 3, 4 and 5.

Discussion. The present observation significantly demonstrates the sensitivity of the early embryo to A., a fact to be considered in chemotherapeutic efforts using this compound as antibiotic or antitumor agent in man. Furthermore, A. like O-Diazo-Acetyl-L-serine and 6-Diazo-5-oxo norleucine(2,3) permits the control of reproduction by either preventing proper implantation of the fertilized ova or destruction of the implanted embryos in the early phases of gestation without severely intoxicating the mothers.

Summary. Alazopeptin had the following toxicity in the adult Long-Evans rat: (1) Single LD_{50} of 150 mg/kg, the 5 LD_{50} of .3 mg/kg. (2) Alazopeptin destroyed *in utero* entire litters of Long-Evans rats (at time of implantation) when given i.p. on 7th and 8th day of gestation in doses of .3 mg/kg. (3) And in doses of .5 mg/kg i.p. (at mid term) when given on 11th and 12th day of gestation. (4) Placental remnants frequently survived the fetuses and were found at term. (5) The lethal dose of A. to the fetus of .5 mg/kg was approximately 1/30 of the LD_{50} for the mother animal.

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Cardiovascular Responses to Norepinephrine in Acute Adrenal Insufficiency.* (23912)

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Since Elliot's observations(1) on responses of adrenalectomized cats to various pressor agents, numerous investigators have concerned themselves with the problem of cardiovascular reactivity in adrenal insufficiency. A summary of the work on cardiovascular responses to sympathomimetic stimuli in adrenal insufficiency appears in the review by Ramey and Goldstein(2). An impairment in the vas-

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TABLE I. Increase of Force of Cardiac Contraction (g) following I.V. Norepinephrine (μg/kg).

Dose	Mean value ± S.D. Control	Adrenx	"p" value
.1	17 ± 15	12 ± 8	>.5
.5	49 ± 31	35 ± 18	>.3
1.0	68 ± 35	57 ± 24	>.4
2.0	75 ± 51	73 ± 28	>.9
3.0	79 ± 50	79 ± 26	>.9
4.0	79 ± 49	81 ± 24	>.9
5.0	80 ± 47	81 ± 24	>.9

cular responses to epinephrine and nor-epinephrine in adrenalectomized animals has been claimed by some investigators(3-5) but denied by others(6,7). Cardiac responses have not been thoroughly studied. The clinical observation of impaired pressor responses to nor-epinephrine in certain cases of traumatic or surgical shock with restoration of responses by adrenal steroid therapy has renewed interest in this problem(8).

Methods. Seven graded doses of norepinephrine were given to each of 5 adrenalectomized and 8 control dogs. The doses ranged from 0.1-5.0 μg/kg i.v. The responses measured were the increases in pulmonary and systemic arterial blood pressures and in force of cardiac contraction. The experimental animals were adrenalectomized *via* the lumbar approach one side at a time. The operations were 5-8 days apart. Measurements were made on these dogs 2 days after the second operation, at which time the animals were less active, anorexic, weak, and less responsive to external stimuli. They had been given some salt in their drinking water after the second stage adrenalectomy but no hormone replacement. Half of the control group were sham adrenalectomized 2 days before making the measurements. They were active, strong, had good appetites, and generally appeared completely normal. Their responses were identical to the other half of the control group which were unoperated and so the two groups have been considered together as the controls. All measurements were made during light pentobarbital anesthesia with the chest open and with respirations maintained by a Starling type pump. Pulmonary and systemic arterial pressures were measured by P-23D Statham pressure transducers connected, *via* short polyethylene cannulae, to a branch of the left pulmonary artery and a femoral artery, respectively. The force of left ventricular contraction was measured by means of an arch strain gauge sutured to the ventricle according to the method of Boniface, Brodie and Walton (9). All recordings were made with a multichannel oscillographic recorder† after suitable amplification. The pressure transducers and arch strain gauge were calibrated for each experiment; their responses were also checked and found to be linear.

Results. The initial systemic arterial pressures were significantly lower ("p" value of <0.01) in the adrenalectomized group of animals (71/35 mm Hg) compared to the control group (121/77 mm Hg). The initial pulmonary arterial pressures were also lower in the adrenalectomized group (9/<1 mm Hg) compared to the control group (15/5 mm Hg); the difference was of borderline significance, however ("p" value between 0.05 and 0.1).

There were no significant differences between the two groups in either their cardiac (Table I) or pressor (Tables II and III) responses to the graded doses of norepinephrine. The *responses* are recorded as the *increases* above the initial values.

TABLE II. Increase of Femoral Arterial Pressure (mm Hg) following I.V. Norepinephrine (μg/kg).

Dose	Mean value ± S.D. Control	Adrenx	"p" value Systolic	Diastolic
.1	26/20 ± 9/ 8	18/14 ± 4/ 5	>.1	>.2
.5	53/39 ± 17/15	46/38 ± 21/18	>.5	>.9
1.0	73/42 ± 18/19	72/57 ± 28/24	>.9	>.2
2.0	110/61 ± 32/17	99/69 ± 32/28	>.5	>.4
3.0	108/72 ± 39/23	105/80 ± 21/16	>.8	>.4
4.0	135/78 ± 41/19	124/80 ± 19/15	>.5	>.8
5.0	142/82 ± 41/20	139/86 ± 22/21	>.9	>.7

† Electronics for Medicine, White Plains, N. Y.

TABLE III. Increase of Pulmonary Arterial Pressure (mm Hg) following I.V. Norepinephrine (μg/kg).

Dose	Mean values	
	Control	Adrenx
.1	1.5/ .5	2/ .4
.5	4.5/1.0	5/2.0
1.0	8 /1.0	10/3.0
2.0	14 /1.0	15/4.0
3.0	17.5/1.0	20/6.0
4.0	18 /1.0	21/5.0
5.0	18 /1.5	22/3.5

In addition, preliminary experiments were done in which dose-response curves were obtained in control and in adrenalectomized dogs before and 2 hours after infusion of hydrocortisone (5-10 mg/kg i.v.) Hydrocortisone did not modify the dose-response curve in either group of animals within this time interval. In 2 of the adrenalectomized animals, autopsies were done and the heart carefully examined both grossly and microscopically. There was no evidence of any coronary artery or myocardial abnormality such as that reported by Hall and Cleghorn(13).

Discussion. The failure to demonstrate impaired pressor responses to nor-epinephrine in the adrenalectomized dogs is in contrast to the findings of Ramey, Goldstein and Levine (10). These investigators found impaired pressor responses to nor-epinephrine (0.25-1.0 μg/kg i.v.) in adrenalectomized dogs maintained 5 to 10 days post operatively on desoxycorticosterone acetate; they found in addition that adrenal cortex extract restored the responses to nor-epinephrine. Impaired pressor responses to epinephrine in adrenalectomized animals was reported by Remington *et al.*(6) and by Cleghorn *et al.*(11). On the other hand, Salmoiraghi and McCubbin(12) found no impairment in the pressor responses to either nor-epinephrine or epinephrine in untreated adrenalectomized dogs, 1-4 days post-operatively. Brown and Remington(7) recently found no impairment in hind limb vascular responses to epinephrine or nor-epinephrine in adrenalectomized dogs as indicated by flow/pressure ratios in the femoral arterial bed. In spite of this, they did observe diminished systemic arterial pressor responses in the adrenalectomized dogs compared to controls bled to approximately the same initial blood pressure levels. These investigators postulated that the diminished pressor responses were the result of impaired cardiac responses. The results of our study fail to show any impairment in left ventricular contractile force responses to nor-epinephrine in adrenalectomized dogs.

We are unable to explain the inconsistencies referred to above. However, the following points can be made toward this end: 1) No 2 of the above experiments were carried out under the same experimental conditions. The duration, and undoubtedly the degree, of adrenal insufficiency varied widely. In some experiments animals were maintained on desoxycorticosterone and/or adrenal cortex extract; in other experiments the animals were untreated. Our own experimental conditions resemble most closely those attained by Salmoiraghi and McCubbin both as to duration (2 days *vs.* 1 and 4 days) of adrenal insufficiency and as to post-operative management (*ad lib.* diet; no replacement therapy); our results also coincide most closely with their results with regard to pressor responses. 2) The selected test dosages of epinephrine and nor-epinephrine vary widely; this point deserves special emphasis since Remington *et al.* (6) found reduced pressor responses to small, but not to large, doses of epinephrine. Few investigators attempted to obtain adequate dose-response curves. 3) In no study has there been adequate simultaneous evaluation of both cardiac and vascular responses.

With respect to the interpretation of our own results the following limitations deserve mention: 1) The pressor responses were measured as the increases in blood pressure above initial blood pressure levels. As pointed out under results, initial blood pressure in adrenalectomized animals was significantly lower than in the controls. The possible influence of variable initial pressure levels on the pressor responses is unknown; successful attempts to control this by making the control animals hypotensive would appear to depend on the way in which hypotension is produced, *i.e.*, it should be produced in a manner which is hemodynamically similar to the mechanism underlying the hypotension in adrenal insuf-

ficiency. 2) Cardiac contractile responses were also measured as increases in the force of left ventricular contraction above the initial level. Technical difficulties with the method precluded accurate comparison of initial contractile force, under conditions of identical fiber length and tension, between the control and experimental groups. 3) All measurements were made during anesthesia, with chest open, and during artificial respiration.

With the above limitations in mind, we conclude the following: 1) the adrenalectomized animals were in mild symptomatic adrenal insufficiency as indicated by general appearance and behavior, hypotension, and decreased tolerance to pentobarbital (the dose required for anesthesia being approximately one-half that required by controls). 2) Systemic and pulmonary hypotension is an early feature of experimental adrenal insufficiency. 3) There is no significant demonstrable impairment in pressor or cardiac contractile responses to norepinephrine in dogs with early (2 days) adrenal insufficiency which are not on replacement therapy. 4) The unimpaired cardiac contractile responses plus unimpaired pressor responses suggest, but do not prove, that the vascular responses to nor-epinephrine are also unimpaired. 5) The results afford no explanation for the hypotension present in adrenal insufficiency. Anatomical lesions in the heart do not necessarily accompany the hypotension.

Summary. Cardiovascular responses to norepinephrine have been studied in adrenalectomized and control dogs. The initial mean systolic and diastolic blood pressures of the adrenalectomized group in both femoral and pulmonary arteries were lower than those of the control group. There was no significant difference between the 2 groups in ability to respond to intravenously injected norepinephrine by an increase in force of cardiac contraction or by an increase in systemic or pulmonary blood pressure. Myocardial or coronary artery lesions did not occur in the 2 adrenalectomized animals examined.

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Retrograde and Forward Perfusion of Isolated Canine Lung Lobes.* (23913)

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The influence of mechanical factors on pulmonary vascular resistance has become established during recent years. Since the pulmonary vascular bed is an easily distensible system, it is not surprising that changes in transmural pressure exert a major influence on resistance to blood flow through this system. Because earlier studies have been made only on forward perfusion of intact or isolated lungs(1-5) it seemed of interest to investi-

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gate the influence of these same mechanical factors during retrograde perfusion of the lung.

Methods. Adult mongrel dogs were anesthetized with intravenous 5% sodium pentobarbital (25-30 mg/kg) and were given intravenous heparin (3 mg/kg body weight). Following the administration of heparin, the femoral artery was cannulated and blood was collected for subsequent use in perfusion of the lung lobes. As soon as the dog was exsanguinated, a right thoracotomy was performed and both the right and left lower lobes were removed. In the resection of the lobes, care was taken to clamp the branches of the pulmonary arteries and veins with bulldog clamps in order to prevent air embolization. The lobes were then weighed in order that flow/gram of pulmonary tissue could be determined. In all of the experiments a cannula was placed in the bronchus and tied in place. Depending on the direction of the perfusion, a cannula was placed and tied into either the major pulmonary vein or artery. In those experiments in which wedge pressures were measured, a fine polyethylene catheter was wedged into the arterial or venous bed prior to the insertion of the perfusing catheter. Right and left lobes were alternated as to direction of the perfusion. The lobe was placed in a methyl methacrylate chamber and perfused with autologous blood through a system from which all glass had been excluded. The perfusing blood was passed through an alleviator shortly after leaving the Sigmamotor pump, in order to decrease the pulse pressure. At the time the perfusion was started, the lobe was ventilated with 100% oxygen at a pressure of 16 cm of water and a rate of 20-25 inflations per minute. Pressures were measured by Statham pressure transducers and recorded on a direct writing Sanborn apparatus. Perfusing rate of flow was determined by calibrating the system prior to the experiments by measuring delivered volume per minute with a stop watch and graduated vessel. Prior to, and at intervals during the experiment, zero pressure levels were obtained. Outflow pressures were measured by placing a fine polyethylene catheter (#090) 1-2 cm into the outflow vessel and suturing it in place in such a fashion that minimal constriction of the vessel was produced. Flow rates were varied from 45 cc/min to 500 cc/min. In general, a single rate of flow was maintained just long enough to allow the pressures to reach a steady state. The flow rate was increased progressively until obvious pulmonary edema, hemorrhage, and congestion occurred, with subsequent deterioration of the lobe. With outflow pressures above 25 mm Hg pulmonary edema and congestion occurred, and therefore only those observations with outflow pressures of 25 mm Hg or less are reported. Resistances were calculated employing the formula

$$R = \frac{\Delta P}{\text{Flow in cc/g/min}}$$

where ΔP is the difference between the perfusing pressure and outflow pressure.

Results. Tables I and II present the data

TABLE I. Venous Perfusions.
Rate of flow (cc/min.).

Lobe #	Wt, g		45	100	200	300	400	500
1	34	R*	23.4	10.8	11.0	10.8		
		OP†	4.0	2.5	4.0	5.0		
		PP†	35.0	35.0	68.0	100.0		
2	32	R	16.9	15.3				
		OP	1.0	10.0				
		PP	25.0	58.0				
3	32	R	19.7	8.6	4.6	3.2		
		OP	5.0	3.0	9.0	17.0		
		PP	33.0	30.0	38.0	47.0		
4	35	R	19.5	10.1	6.6	5.6	5.3	5.1
		OP	0	1.0	2.0	2.0	1.0	1.0
		PP	25.0	30.0	40.0	50.0	62.0	75.0
5	46	R	4.1	6.0	3.2	2.8		
		OP	10.0	12.0	16.0	22.0		
		PP	14.0	25.0	30.0	40.0		
6	30	R	8.7	7.5	3.7	4.0		
		OP	12.0	15.0	25.0	25.0		
		PP	25.0	40.0	50.0	65.0		
7	31	R	12.4	8.4	6.2			
		OP	7.0	10.0	10.0			
		PP	25.0	37.0	50.0			
8	24	R	4.3	3.2				
		OP	12.0	22.0				
		PP	20.0	35.0				
9	25	R	7.2	7.5	4.8	2.5	2.4	1.8
		OP	12.0	10.0	12.0	25.0	22.0	25.0
		PP	25.0	40.0	50.0	55.0	60.0	60.0
Avg		R	12.9	8.6	5.7	4.8	3.9	3.5
		OP	7.0	9.5	11.1	16.0	11.5	13.0
		PP	25.2	36.7	46.6	59.5	61.0	67.5

* mm Hg/cc/g/min. † mm Hg.
R = Resistance. OP = Outflow pressure. PP = Perfusion pressure.

TABLE II. Arterial Perfusions.
Rate of flow (cc/min.).

Lobe #	Wt, g		45	100	200	300	400	500
1	34	R*	18.2	8.5	5.7	5.5	5.7	
		OP†	2.5	7.5	9.0	15.0	20.0	
		PP†	27.0	32.5	42.0	63.0	87.0	
2	27	R	19.8	14.9	10.0	7.7		
		OP	2.0	5.0	5.0	7.0		
		PP	35.0	61.0	79.0	92.0		
3	37	R	28.6	14.8	8.3	6.4	5.9	
		OP	2.5	3.5	5.0	8.0	10.0	
		PP	38.0	44.0	50.0	60.0	72.0	
4	35	R	11.7	12.9	6.6	4.4	3.9	3.4
		OP	0.0	0.0	1.0	1.0	1.0	1.0
		PP	15.0	37.0	39.0	39.0	45.0	50.0
5	66	R	8.8	6.6	4.7	4.0	3.9	3.7
		OP	5.0	10.0	11.0	12.0	13.0	12.0
		PP	11.0	20.0	25.0	30.0	37.0	40.0
6	46	R	30.6	18.3	12.4	8.9	9.0	8.9
		OP	0.0	0.0	1.0	4.0	2.0	2.0
		PP	30.0	40.0	55.0	62.0	80.0	100.0
7	31	R	11.7	9.3	7.1			
		OP	1.0	10.0	9.0			
		PP	18.0	40.0	55.0			
8	31	R	13.1	9.6	5.1	3.6	2.9	
		OP	11.0	14.0	15.0	20.0	22.0	
		PP	37.0	45.0	48.0	55.0	60.0	
9	29	R	4.5	6.4	4.1			
		OP	18.0	18.0	22.0			
		PP	25.0	40.0	50.0			
Avg		R	16.3	11.3	7.1	5.8	5.2	5.3
		OP	4.7	7.6	8.7	9.6	11.3	5.0
		PP	26.2	39.5	49.2	57.3	63.5	63.3

* mm Hg/cc/g/min. † mm Hg.

R = Resistance. OP = Outflow pressure. PP = Perfusion pressure.

from 9 pairs of lower lobes, in which corresponding numbers in the 2 tables represent lung lobes from one dog. It will be noted that the average perfusion pressure necessary to obtain a particular flow rate is not greatly different as between retrograde and forward perfusion. However, the average values of outflow pressure are higher in the retrograde perfusion situation and therefore the calculated resistances to flow between the points of measurement are somewhat higher for forward than for retrograde flow. It will also be noted that the resistance falls with increasing perfusion pressure with both forward and retrograde flow. Within limits of accuracy of measurement this was the case in all instances but two, in which outflow pressures were high at low flows. A comparison between outflow pressure levels and pulmonary vascular resistance indicates that maximal resistance was noted when outflow pressures were minimal. This correlation was observed with both retrograde perfusion and forward perfusion.

Discussion. It is of interest that the resistance to retrograde flow of blood in the pulmonary circuit is no greater, and may be somewhat less than that for forward flow within the small vessel bed. A further analysis of the relative importance of venous and arterial bed distention under pressure upon resistances to flow in the two segments must await more detailed analysis of pressure gradients within the vascular bed, including arterial wedge pressure measurements. It would be of interest to know the volume elasticity of arterial and venous segments and the relative effects of increasing the diameters of arterial and venous segments by pressure changes upon the resistances to blood flow through them.

Precise numerical data have not been obtained in this study, but it has been noted quite regularly in this study that pulmonary edema occurs sooner at a given high flow rate with retrograde than with forward flow. This would indicate, as would be predicted, that venules offer less resistance to distention than do arterioles and that therefore a larger fraction of the perfusion pressure obtains in the capillaries with retrograde than with forward flow. The occasional occurrence of low resistance at low flow rates could perhaps be related to variations in the magnitude of arterio-venous shunts(6,7) in one animal as compared to another.

Summary. Retrograde perfusion of the lung vessels of the dog is accomplished with no more resistance to flow than in forward flow. The calculated resistances in the smaller vessels is on the average less with retrograde than with forward flow. The resistance to flow in either direction diminishes with increased perfusion pressure. Gross observation indicates a more rapid production of lung edema at high perfusion rates with retrograde than with forward perfusion.

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Methods for Studying Lymphatic Function in Intact Man Utilizing Au^{198}.* (23914)

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Methods have been devised for studying functional patterns, dynamics and quantitative particle pickup by intact lymphatics and lymph nodes under various conditions. Previous methods using intracutaneous injection of particulate dyes, as that of Hudack and McMaster(1), have demonstrated the pattern of fine superficial cutaneous lymphatics in various experimental and clinical states. Demonstration of deeper lymphatic channels and lymph nodes by pickup of stained particles or radioactive gold has required operative exposure of lymph-bearing tissues and lymph nodes(2,3). The methods to be described require no operation and provide graphic records of lymph node pickup, and quantitative measurements of such pickup. Thus, serial physiologic and diagnostic studies can be performed.

Method. Pictorial pattern of lymph node pickup is obtained by local injection of radioactive gold and the use of a scintiscanner. Similar Lymphatic Scintigrams were first obtained in dogs(4). Injection of Au^{198} into various sites in head and neck precedes continuous or interval scintiscanner recording of the pickup by deep lymphatics and lymph nodes. Since the half-life of radioactive gold is 2.69 days, speed of disappearance from site of injection and later distribution in the lymphatic system can be recorded. Lymphatic scintigrams have now been obtained in both upper and lower extremities in intact humans. Fifty to 100 microcuries of radioactive gold are injected into subcutaneous tissues of calf of leg. Deposition in femoral and inguinal nodes within 10 minutes to an hour can be detected under normal conditions. The first node (Fig. 1, part a.) to be outlined is usually in the femoral triangle about one to two inches below the inguinal ligament. The pictorial pattern obtained can be varied by altering the sensitivity and kinetics of the scintiscanner. This counter moves automatically across and up and down the field at varied speeds which increase or decrease the concentration of recorded dots. Thirty to 60 minutes may be required to scan the area

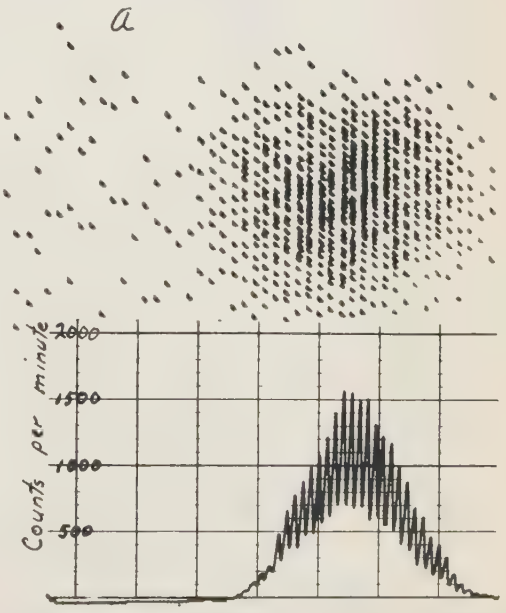


FIG. 1. a. (top) Lymphatic scintogram of femoral region 24 hr after inj. of 100 microcuries of Au^{198} inj. into subcut. tissues of calf. b. (bottom) Count rate meter graph run simultaneously and coordinately with 1-a.

* Research project supported by U. S. Public Health Service Grant.

completely. Spacing can also be varied to delineate lymph node pickup. Concentration of recorded counts can be varied by the scintillation crystal used and size of collimator opening. Anatomic delineation of individual lymph nodes in the pattern is aided by a pulse height analyzer which records the peak energy of gamma rays. The distance of crystal from lymph nodes influences the pickup. Finer delineation of individual lymph nodes may be obtained by introduction of a photographic circuit. Some background effect can be eliminated by this means.

Two methods have been used for recording pickup in individual lymph nodes quantitatively. Numerical counts can be made with the scintillation counter and recorded as a grid directly from the field to be investigated, at spacings of one centimeter or more. A count rate meter and automatic recording graph will provide a continuous time-record of pickup by individual lymph nodes (Fig. 2). This is an effective method of obtaining information concerning the dynamics as well as quantity of lymph node pickup. Counts/minute may be recorded continuously over many hours, or at intervals for days.

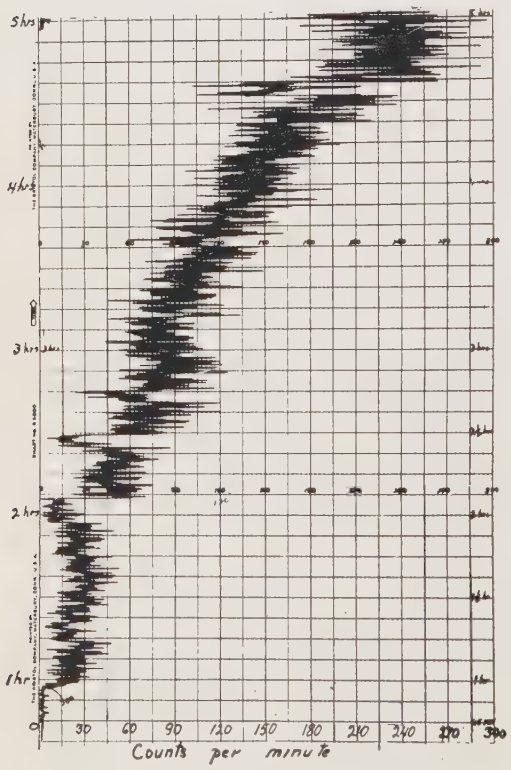


FIG. 2.

The count rate meter, recording graph, and scintiscanner records can be run simultaneously. In this way a quantitative estimate of lymph node pickup and a pictorial representation is obtained. Timing of the devices can be arranged so that records can be interpreted coordinately when attached to each other (Fig. 1, b).

Various anatomic, physiologic and pathologic factors alter the lymphatic pattern obtained, the dynamics and quantity of lymph node pickup. Choice of isotope, dosage and particle size, and volume and pressure of injected material, are important in establishing baseline conditions. Baseline conditions used were 1/10th cc of injected material containing 50 to 100 microcuries of Au^{198} of .003 average particle size. A photograph of particle size of material can be obtained with an electron microscope. Rate and amount of pickup depend on size, dosage, volume and pressure. Rate of pickup also depends on site of injection, since skin, subcutaneous and deeper lymphatic systems vary in their size, capacity and function. Some physiologic and pathologic states are being studied.

Summary. Methods have been devised, using Au^{198}, for studying functional patterns, dynamics and quantitative pickup of lymphatics and lymph nodes. These can be applied to intact man, as well as animals, under varied conditions.

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24-Hour Rhythms at Several Levels of Integration in Mice on Different Lighting Regimens.* (23915)

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Ordinarily, lighting regimen controls the timing of eosinophil rhythm(1), as well as activity rhythm(2), in mice and other experimental animals(3,4). A shift in timing of daily periods of light and darkness can therefore be used to displace the daily time of peak or trough of the eosinophil rhythm(3), as long as physiologic conditions are maintained [*e.g.*, in fully-fed, healthy animals, but not in mice severely restricted in dietary calories(1)]. It will be shown that daily "high" and "low" of other 24-hour rhythms also may be shifted by manipulation of illumination. An earlier suggestion, that lighting is ordinarily the dominant synchronizer of eosinophil rhythm in mice(5) can now be extended to a number of additional 24-hour periodic phenomena, some of them metabolic and/or cellular functions, others reflecting operation of integrative controls(3,6) of rhythm.

Materials and methods. Mice of inbred strains, maintained in the Division of Cancer Biology at the University of Minnesota, by brother-to-sister mating, for over 50 generations were used: D_8 (Dilute Brown) mice for the study of epidermal mitoses (males, ~ 4 months of age), of liver glycogen (both sexes, ~ 7 months of age) and of blood "corticosterone" (females, ~ 15 months of age); C (Bagg Albino) mice for the study of liver mitoses (males, ~ 5 weeks of age), of liver DNA (both sexes, ~ 5 weeks of age), of liver phospholipid and RNA (males, ~ 6 weeks of age), and of blood sugar (males, ~ 2½ months of age). From the time of weaning and throughout the investigations, Purina Laboratory Chow and tap water were available to the animals *ad libitum*. The mice were first "standardized" for at least one week prior to each study. For this purpose, they were placed in single cages, in rooms maintained at a temperature of 78 ± 2°F and illuminated by artificial light only. The mice were disturbed only once weekly for cage cleaning and food replacement. The cages were last cleaned and fresh food was given 3 days prior to killing. Thereafter, nobody entered the room, except for a daily brief and quiet checking of the water bottles and, in the experiments involving radiophosphorus, for tracer injection, 2 hours before killing, as described(7). Metal cages admitting light solely through the galvanized mesh door were used. Prior to study, photometer readings were made during the light period in selected cages, from different positions on the racks. On the top rows, light intensities varied from the front to the back of a given cage, from ~ 14 to ~ 3 footcandles; on the bottom rows the corresponding variation was from ~ 3 to ~ 1 footcandles. In each room, a clock-controlled switch turned the lights on and off, at 12-hour intervals, according to a set schedule. Two schedules of illumination were used: the "regular" schedule provided for light from 6 a.m. to 6 p.m., while the "reversed" schedule provided for light from 6 p.m. to 6 a.m. All of the mice were first placed on the regular schedule for the week of standardization. Thereafter, in the studies of blood glucose, liver glycogen and phospholipid, the regular schedule was continued for some of the mice, while others were placed on the reversed schedule. Either schedule was then maintained unchanged for 8 days (phospholipid) or for 2 weeks (blood glucose and liver glycogen). Finally, the mice on both schedules were killed on the same day, at the same 2 times which were those of the daily "high" and "low" in a given variable (previously established on a regular schedule of lighting, in experiments based upon data from 7 groups of mice, killed at 4-hour intervals, during each of 2 independent 24-hour periods) (8,9, and unpublished data). In these studies

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the groups on the 2 schedules of lighting, were actually killed in succession, at each of the 2 times of sampling—the hours listed with the results representing the mid-points of the period needed for the killing of both groups (the regular and the reversed). This total period of sampling was of about one-hour duration, mice in a given group being killed at 2-minute intervals. Mitotic activity in pinnal epidermis and hepatic parenchyma, and hepatic RNA and DNA metabolism were studied at 2 time points, with mice on a reversed schedule for 8 days (hepatic mitoses and RNA), 2 weeks (DNA) and 23 days (pinnal mitoses), respectively. Earlier data for comparable mice, kept on the regular schedule will be used for comparison(8,9 and unpublished data). The two series on serum corticosterone each describe 7 groups of mice, killed at 4-hour intervals, throughout a 24-hour period; in this study, the group on the regular schedule was sampled first. For the remainder of the mice, the reversed schedule was then instituted and maintained for 2 weeks prior to killing. Moreover, for the study of "corticosterone," pools of serum were used, by contrast to the rest of the data, all of which describe individual mice. Methods described elsewhere were used for determinations of blood glucose(10), blood "corticosterone" (according to Silber, Bush and Oslapas), liver glycogen(11), and relative specific activity (RSA) of hepatic phospholipid (7), RNA(12), DNA(13). The tissues processed for mitotic studies were fixed in Bouin's solution, embedded in paraffin, cut at 5 μ, stained with Heidenhain's iron hematoxylin and mitotic counts were made as previously described(14), all of the counts in a given study being done by the same individual.

Results. The results of this study are shown in the figures, which reveal considerable variability in individual values (height of columns) and/or the standard error of each mean (see shaded areas around means, indicating $\pm$ 1 standard error). The limitations of our illumination which varied from the top to the bottom of the racks as well as from the front to the back of a given cage (see methods), may have contributed substantially to this variability. It is further recognized that with the "spot-checks" carried out herein at only 2 time points on the reversed schedule, the completeness of inversion of rhythm cannot be ascertained, a consideration which may be particularly pertinent to the data on hepatic mitoses (Fig. 1f). We can conclude, however, for all of the functions studied that an important shift of rhythm had taken place following the shift of lighting schedule. It also is pertinent in this connection that except for blood "corticosterone," the day-night differences on both schedules were tested by Student's t and were found to be significant. Blood "corticosterone" is the only variable for which a statistical test was not carried out, since each point, in each series, is based upon only one determination, made on a pool of serum. It is pertinent, though, that the peak in blood "corticosterone" occurred at the time previously predicted on the basis of the daily periodic eosinophil behavior of mice recorded by others as well as ourselves on a regular (15-18) or on a reversed schedule(1). While the occurrence of the daily "corticosterone" peak at the time of periodic eosinopenia suggests that it is real, it is noted that the degree of specificity of the fluorometric procedure used for corticosterone determination has not been ascertained in this study. Therefore, instead of plotting absolute amounts, the relative levels of blood "corticosterone" are given in Fig. 1 d, *i.e.* the changes are expressed as per cent of their 24-hour mean.

With particular reference to Fig. 1 f, it may be noted that the day-night differences in mitotic count of liver parenchyma had changed sign and were statistically significant, at 8 days after inversion of lighting, while at the same time point, mitoses in pinnal epidermis were as yet not reversed (data not graphed). But at 23 days after lighting inversion, the shifted timing of mitoses in pinnal epidermis was clearly apparent (Fig. 1 f). That a shift of rhythm occurs only gradually (rather than abruptly) following the sudden change in lighting regimen has previously been shown elsewhere for rhythms at the level of the body as a whole(2,4) and in our laboratory, with special reference to cellular rhythms(1,19). This study, in turn, demonstrates that such shifts of rhythm (by shifts of lighting schedule) are the rule rather than the exception, as

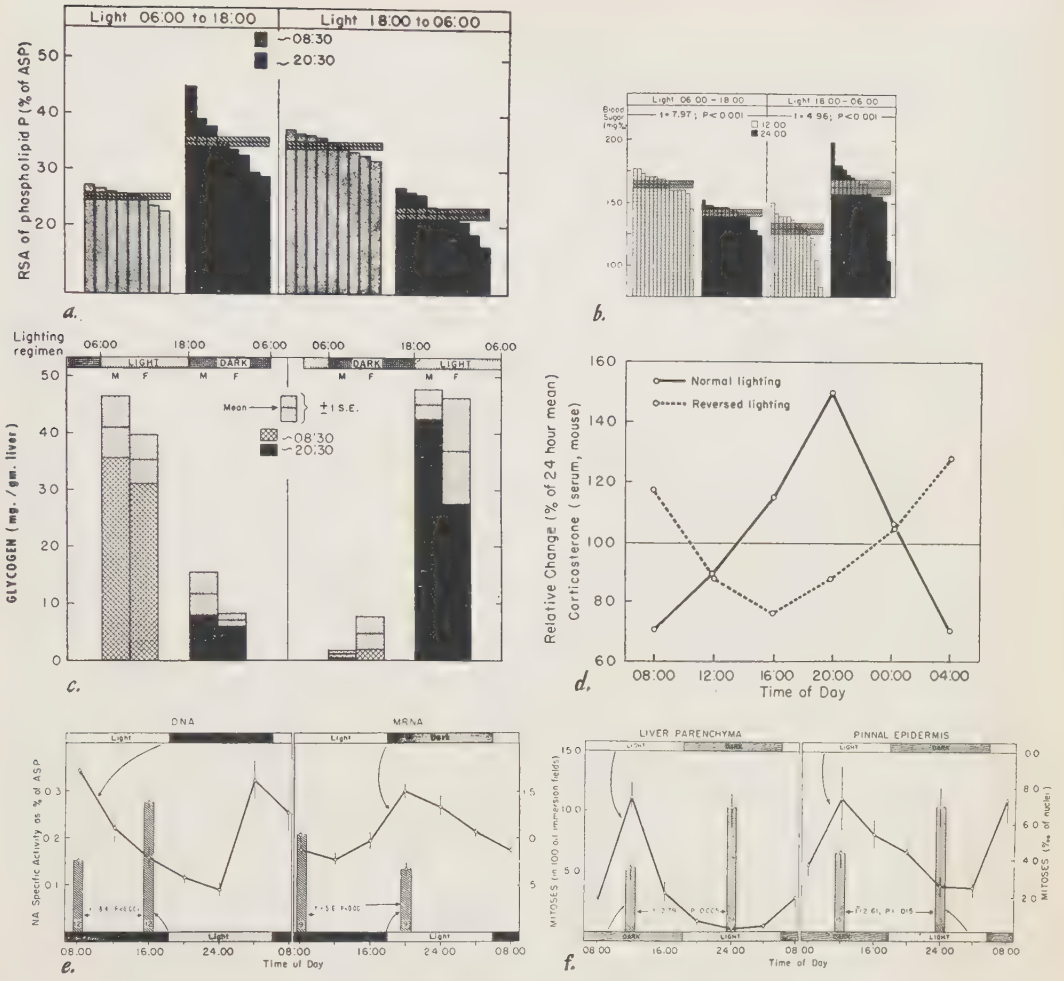


FIG. 1. Physiologic 24-hr rhythms at various levels of organization. Mouse. Data on schedule of light from 6 p.m. to 6 a.m., alternating with 12 hr of darkness, are compared with data on light from 6 a.m. to 6 p.m. Previously published data are used for lines in Fig. 1e(8). t value for day-night difference in epidermal mitoses based upon $\log_{10}$ transformation of counts.

regards 24-hour periodic phenomena at different levels of organization (20-33).

Summary and conclusions. The following variables were studied in the mouse under conditions standardized for evaluation of 24-hour periodicity: glucose and "corticosterone" in blood; mitoses in pinnal epidermis and hepatic parenchyma; liver glycogen and the hepatic metabolism of cytoplasmic phospholipid and ribonucleic acid and of nuclear deoxyribonucleic acid. For each variable, data obtained with light from 6 p.m. to 6 a.m. alternating with 12 hours of darkness, were compared with data obtained with light from 6 a.m. to 6 p.m. A shift in timing of 24-hour rhythms, following the inversion of lighting regimen came clearly and consistently to the fore. Ordinarily, environmental lighting is the dominant synchronizer of various 24-hour rhythmic functions, at different levels of organization of mice: by instituting an appropriate schedule of lighting and by allowing thereafter for the necessary shift time, one may set the peak or trough of rhythm to any chosen clock hour. Moreover, by controlling the timing of various periodic functions with respect to the environment, defined phase relations are brought about among the various physiologic rhythms themselves. The possibility to control timing of these rhythms by an

easily manipulated environmental factor is of obvious practical interest, but it also constitutes a requisite for a basic analysis of functions, within the range in which they vary under ordinary circumstances.

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Staphylococcal Enterotoxin: Increased Vomiting Incidence in Monkeys Following Subemetic Doses of Dihydroergotamine.* (23916)

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Very little definitive information is available on mode and site of action of staphylococcal enterotoxin. It has been suggested that action of enterotoxin is peripheral rather than central and that it affects either the sensory nerve endings or the smooth muscle of small intestine(1). Experiments with isolated intestinal strips show no direct stimulatory action of enterotoxin (unpublished, 2). The action of enterotoxin upon some specific area of the central nervous system has also been suggested(3). The results with animals subjected to various denervation and surgical procedures have been interpreted as indicating the site of action in the forebrain(4), but enterotoxin induced vomiting in chronic, totally decorticate cats with the third ventricle and surrounding structures intact(5). The

* This work was supported by contributions from various companies and associations of food industries.

rapid "neutralization" of toxic manifestations in pseudomembranous enterocolitis by corticotrophin (ACTH) is indicative of a relationship between enterotoxin and the adrenal cortical hormones(6). The small, but definite, elevation of serum glutamic oxalacetic transaminase of monkeys following oral administration of enterotoxin suggests cellular damage at some site (to be published).

The present communication is concerned with attempts to influence by administration of dihydroergotamine methanesulfonate (DHE-45) and other drugs the incidence of emesis in monkeys fed enterotoxin. Should a compound decrease the incidence of vomiting, then a reasonable basis is provided for investigating the possibility that the drug and enterotoxin had opposite pharmacological activities. Conversely, should a drug treatment increase the number of monkeys which vomit, the possibility would exist that the drug and the enterotoxin had similar pharmacological actions. Experiments of this nature may provide a clue to mode and site of action of staphylococcal enterotoxin.

Materials and methods. Partially purified enterotoxin preparations dissolved in 50 ml of water were fed to *Macaca mulatta* by stomach tube. Vomiting in these monkeys within a 5 hr period was considered a positive response to the enterotoxin. The animals were employed in groups of 6. The control group received toxin alone and the test group received the same dose of same toxin preparation in addition to the drug being tested. Since monkeys were fed enterotoxin samples several times and varied considerably in their sensitivity to enterotoxin, the animals for each experiment were selected so the reactivity to enterotoxin of the test and control groups of monkeys were as nearly equal as possible(7). When monkeys were reused in experiments involving the same drug, they were placed as equally as possible in both control and test groups. The enterotoxin samples were derived from 3 enterotoxigenic strains of *Staphylococcus*. Toxin preparations were in varying stages of purification. Most feeding experiments were performed with relatively crude concentrates, but one experiment was done with a preparation of 20-25% purity(8). The dosage of toxin used was one considered capable of inducing emesis in one to 3 of the 6 control animals used in that experiment. Relatively large amounts of drugs were employed to accentuate any differences in responsiveness of monkeys to enterotoxin. If the drug had emetic activity of its own the dosage used was one which was subemetic.

TABLE I. Effect of Dihydroergotamine Methanesulfonate on Vomiting in Monkeys Fed Staphylococcal Enterotoxin.

Enterotoxin		No. vomiting/No. tested		
Strain	mg/monkey	Control*	Test†	Drug‡
196	11.4	3/6	5/6	0/1
"	"	1/6	"	0/6
"	2.3	2/6	3/6	
"	1.14	3/6	5/6	
"	.515	2/6	3/6	
"	4.6	1/6	4/6	
"	"	"	2/6	
8	17.68	3/6	6/6	0/6
S–6	24.0	2/6	4/6	"
"	.05	1/6	3/6	"
	Totals	19/60	40/60	0/25

* Enterotoxin alone. † DHE-45, 1.5 mg subcut. + enterotoxin. ‡ DHE-45, 1.5 mg subcut.
χ^2 probability less than .01.

Results. The number of monkeys vomiting in the group treated with dihydroergotamine methanesulfonate (DHE-45) was approximately twice that of control group receiving enterotoxin alone. DHE-45 was injected subcutaneously in dose of 1.5 mg/animal (average wt about 3.5 kg) 45-60 minutes before the toxin was fed. A total of 60 monkeys in both control and test groups was used. None of 25 monkeys vomited when the same quantity of DHE-45 was given subcutaneously and 50 ml of water was fed. The results are presented in Table I.

The data of Table I were obtained through oral administration of enterotoxin prepared from 3 strains of *Staphylococcus*. The increase in vomiting incidence with the toxin from all 3 strains is of interest since there is evidence that enterotoxin from different strains is antigenically different(7,9).

No significant difference in incidence of vomiting in monkeys fed enterotoxin was observed in a limited number of similar experiments involving other types of drugs. Diphenhydramine (benadryl) was used for its antihistaminic effect; apomorphine because

its site of emetic action has been established. Veriloid (alkaloid of *Veratrum viride*) was included since the site of emetic action of the veratrum alkaloids is not the central nervous system but seems to be associated with the nodose ganglion(4). The possible effect of ganglionic blockage was tested by use of tetraethylammonium chloride (Etamon chloride). Atropine was employed as an example of a parasympathetic nervous system depressant. The possible prophylactic effect of the adrenal cortical hormones (in the form of adrenal cortical extracts) was investigated because ACTH has been reported to alleviate the toxic symptoms of pseudomembranous enterocolitis(6).

Since ergot alkaloids and their dihydrogenated derivatives possess emetic property, the possibility that increased incidence of emesis among DHE-45 treated animals might be due to summation of 2 similar pharmacological activities was investigated. So far no adrenolytic activity of enterotoxin as exhibited by DHE-45(10) has been found. Enterotoxin does not inhibit relaxing effect of epinephrine on isolated small intestine of the rabbit and does not affect the action of epinephrine in antagonizing the contraction of the rat uterus stimulated by acetylcholine. Enterotoxin does not inhibit the hyperglycemia in rabbits which follows subcutaneous injection of epinephrine. No distinctive blood pressure change in cats under chloralose anesthesia has been observed following injection of enterotoxin samples.

DHE-45 is also a serotonin antagonist(11). Serotonin antagonism can be demonstrated with some enterotoxin samples on the isolated oestrous rat uterus preparation, but this reaction does not seem specific to the enterotoxin itself.

Discussion. The number of monkeys which vomit after oral administration of staphylococcal enterotoxin is significantly increased by subcutaneous injection of subemetic doses of dihydroergotamine methanesulfonate. Since only partially purified preparations of enterotoxin were used, it is possible that the agent responsible for the observed results may be some substance other than the enterotoxin. However, the monkey feeding test is the most specific of the tests for detection of enterotoxin in untreated crude staphylococcal culture filtrates. Presumably, substances other than enterotoxin present in these filtrates are rendered innocuous or are not absorbed; they do not provoke emesis. Thus, it is probably the enterotoxin itself which plays a role in increasing the incidence of emesis in monkeys treated with subemetic quantity of DHE-45.

The results suggest a summation of effects of 2 emetic compounds. The subemetic quantity of DHE-45 may give a stimulus below the threshold necessary to evoke vomiting; the enterotoxin may give a similar subthreshold stimulus in animals which do not vomit with enterotoxin alone. The sum of these 2 subthreshold stimuli may then be sufficient to produce the emetic response. Available information is insufficient to consider possible potentiation effects.

10 μg of essentially pure enterotoxin cause vomiting in monkeys after oral administration (8). This high toxicity and symptoms of staphylococcal food poisoning suggest the nervous system as site of action. If it is a central nervous system toxin, the passage through the blood-brain barrier of a polypeptide of molecular weight of 20-25,000 must be considered. On the other hand, if the results reported in this paper are due to summation of effects of 2 compounds acting on the same site, then the site of emetic action of enterotoxin may be the medullary emetic chemoreceptor trigger zone. This suggestion follows from the demonstration(4) that dogs with chronic ablation of medullary emetic chemoreceptor trigger zone do not vomit after intravenous injection of Hydergine, a mixture of dihydrogenated ergot alkaloids.

A practical application of the observations with DHE-45 may be possible. Repeated feedings of enterotoxin to monkeys result in development of resistance to the toxin. This resistance may become so great that the animals are no longer usable for experiments with practicable doses of enterotoxin. Since subemetic quantity of DHE-45 increases the number of positive reactions in the partially resistant monkeys, a greater number of feeding tests on them may become possible by use of the drug.

Summary. Subcutaneous injection of sub-

emetic dose of dihydroergotamine methanesulfonate increases the number of *Macaca mulatta* which vomit following oral administration of staphylococcal enterotoxin. The possibility that increased incidence of vomiting might be due to summation effects of 2 similar pharmacological compounds has been considered, but no adrenolytic effect of enterotoxin corresponding to that of DHE-45 has been found. The other drugs tested have not significantly affected the incidence of emesis in monkeys following feeding of enterotoxin.

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Food Utilization and Weight Gain in Hypophysectomized Rat as Function of Handling. (23917)

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Previous studies have shown that normal young rats handled or gentled by the experimenter gain significantly more weight and length than unhandled control animals(1-9). Furthermore, these effects of handling appear to be a function of better food utilization rather than increased intake(4,6,7). The literature on this subject has recently been reviewed in detail(10).

This paper reports the effects of handling on weight gain, skeletal growth, food utilization and body composition in the young hypophysectomized rat. It is the first of a series of investigations designed to study the mechanism of various effects of handling in experimental animals.

Methods. Twenty-eight male hypophysectomized rats of Sprague-Dawley strain, obtained from Hormone Assay Laboratories, (Chicago), were used. Hypophysectomy was performed on nineteenth day of age. Animals were weighed and then divided at random into 2 equal groups: control and experimental. Beginning on twenty-first day of age, each animal of experimental group was handled individually by one of us (L.B.) for 10 minutes every day at the same time for 6 weeks. Handling consisted of holding the animal in one hand, and stroking it with the other from head to tail repeatedly, with animal facing experimenter. Control animals were never touched after their original placement in cages. A weighed amount of food in petri dish was placed in each cage daily and leftover food weighed daily to determine daily food consumption. The diet used was of the following content and proportion: 480 g canned dog food (Pard®), 2 slices of crumbled whole wheat bread, 480 ml skim milk, and 2.5 ml Vi-Daylin®* (a liquid multi-vitamin preparation). These ingredients were thoroughly mixed and proved suitable for these animals (tender-jawed following hypophysectomy), and food loss by spillage from petri dish was

* Kindly supplied by Mr. V. Yeager, Abbott Laboratories, N. Chicago, Ill.

TABLE I.

		Weeks							Significance
		0	1	2	3	4	5	6	(p)
Wt (g)	U	38.6	51.3	63.1	69.0	73.2	81.3	89.2	.05
	H	38.6	51.3	63.6	69.9	74.9	84.1	92.8	
Food consumption (g)	U		163.0	235.8	272.0	290.0	319.0	314.4	.05
	H		153.9	225.6	265.0	283.9	306.4	302.8	
Food utilization									
Food consumption (g) / Wt gain (g)	U		12.9	16.2	22.1	27.8	30.0	31.6	.001
	H		12.2	15.2	20.6	25.6	27.2	28.3	
Body composition									
% protein	U							21.1	.6
	H							21.5	
% fat	U							9.1	.5
	H							9.8	
Skeletal growth									
Tibial epiphyseal cartilage (μ)	U							151.5	.9
	H							150.8	

U = Unhandled rats. H = Handled rats. All figures are mean values. Significance values are calculated from data at 6 wk.

minimum. Five % glucose was supplied as drinking water. All animals survived the 6 week experiment and all appeared in good condition throughout the period. The animals were housed individually in wire cages measuring 20 x 17 x 23.5 cm. Cages were arranged in rack so that handled and unhandled animals alternated, and with equal numbers of each group at each level in the rack. The animals were weighed weekly in their cages and weight of cage subtracted so that no handling was involved in the process. The temperature of the animal room was maintained by thermostat at 80 ± 2°F. At end of experiment the animals were sacrificed by gas inhalation and whole carcasses analyzed for protein and fat content. Carcasses were ground in meat grinder and dried to constant weight. Fat was extracted repeatedly with ether, then dried, and weighed. Total protein was obtained by multiplying total nitrogen (macro-Kjeldahl) of dry carcass by 6.25. Skeletal growth was measured by means of the rat tibia test(11), used for assay of pituitary growth hormone.† The sellas of the 5 heaviest animals of each group were removed for histological study and completeness of hypophysectomies was confirmed in all animals.

† These assays were performed in Research Laboratories of Frank Horner Ltd., Montreal, through the courtesy of Dr. L. Mitchell.

Results. Table I summarizes data on weight gain, food consumption, carcass analysis and tibial epiphyseal measurements of the 2 groups of rats. Weight gain of handled group was significantly ($p = .05$) greater than that of unhandled group. The handled animals consumed significantly ($p = .05$) less food than unhandled ones. When a ratio of grams of food consumed/g weight gained is computed, the difference between the 2 groups of rats becomes much more significant ($p = .001$). Another method of demonstrating this difference between the 2 groups of rats is by analysis of covariance(12), in which weight gains may be adjusted for variation in amounts of food ingested. The computed value F (26.5) is significant beyond the 0.1% level of confidence.

Carcass analysis for percentage of fat and nitrogen showed no significant difference between the 2 groups of animals. The same was true of tibial epiphyseal measurements.

Discussion. The results indicate that handling has a significant enhancing effect on weight gain of young hypophysectomized rat. The magnitude of response was considerably less than that previously reported in the young normal rat(1,3,4,5,6,7,8,9). This is probably due to limitations imposed by lack of pituitary hormones necessary for normal growth and development. It is noteworthy

that increased weight gain in hypophysectomized animals was achieved despite a significantly smaller quantity of food ingested. When weight gains are corrected for amount of food ingested, increases in weight of the handled over unhandled group are greatly magnified. In the normal rat, handling has no influence on amount of food ingested(6). The findings indicate that handling increases efficiency of food utilization (increased intestinal absorption and/or diminished metabolism) and that this effect is not dependent upon presence of pituitary or adrenal hormones.

It is apparent that increased skeletal growth previously reported in the handled normal young rat(6), is abolished by hypophysectomy. This suggests that the effect of handling on skeletal growth requires pituitary hormones, presumably growth hormone and/or thyrotropic hormone.

It is pertinent to consider the contribution of handling to weight gains previously reported in hypophysectomized rats treated with insulin(13) or force-fed(14). Since these studies involved extra handling of experimental animals as compared with control animals, it is conceivable that weight gains observed were due in part to extra handling.

Summary. Handling in young hypophysectomized rats resulted in significantly increased weight gain despite ingestion of significantly less food. No effect on skeletal growth or percentage of carcass protein and fat was observed. Increased weight gain in the handled hypophysectomized rat is due to greater efficiency of intestinal absorption of food and/or diminished metabolism rather than a direct stimulation of growth or increased food ingestion.

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Pyridoxal Phosphate (B_6-al-PO_4) Levels in Organs, Leukocytes and Blood of Rats with Developing Vit. B_6 Deficiency.* (23918)

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Recently a method has been described for determination of pyridoxal phosphate (B_6-al-PO_4), the coenzymatically available active form of Vit. B_6, in whole blood and isolated leukocytes(1). Using this technic we estimated the B_6-al-PO_4 content in the only readily accessible prototype tissue, *i.e.,* leukocytes in blood of healthy, nonpregnant controls, mothers at term, and in cord blood of their babies(2). It was found that the pyridoxal phosphate levels of circulating leukocytes were significantly lower in pregnant women than in nonpregnant controls. Since cord blood showed the highest levels, it was concluded that the growing fetus can successfully compete with the mother for this impor-

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TABLE I. Pyridoxal Phosphate in Organs and Blood of 15 Control Rats Fed a Complete Synthetic Diet *Ad Lib.*

Organ wt			Concentration							
			Heart, γ/g		Liver, γ/g		Kidney, γ/g		Muscle, γ/g	
Heart	Liver	Kidney	Mean	Range	Mean	Range	Mean	Range	Mean	Range
.60	7.28	1.49	.82 ± .09	.56–1.35	.86 ± .10	.65–1.80	.93 ± .06	.56–1.70	1.15 ± .21	.72–2.1

Total content			Blood					
			Leucocytes, mγ/mill.		Plasma, mγ/cc		Whole blood, mγ/cc	
Heart, γ	Liver, γ	Kidney, γ	Mean	Range	Mean	Range	Mean	Range
.49 ± .03	6.74 ± .48	1.14 ± .12	1.34 ± .13	.76–2.78	116.4 ± 24.14	84.0–245.4	122.6 ± 19.2	73.0–232.5

tant vitamin and that during pregnancy there exists a relative B_6 deficiency. To substantiate this conclusion it was deemed desirable to investigate whether the pyridoxal phosphate content of circulating leukocytes indeed reflects the Vit. B_6 stores of the mammalian body. Therefore pyridoxal phosphate levels were determined in various tissues as well as in leukocytes, plasma and total blood in young rats on a synthetic Vit. B_6 deficient diet and simultaneously in control rats that had been kept on the same synthetic diet with added Vit. B_6.

Material and method. Young male rats of Wistar strain, weighing about 120 g were placed on a synthetic diet obtained from Nutritional Biochemicals Corp., Cleveland, O., which contained 18% casein supplemented with all essential vitamins with the exception of Vit. B_6. Control animals received the same formula to which had been added 2.2 mg pyridoxin hydrochloride/100 g of diet. Groups of rats on the deficient and supplemented diet were sacrificed after 1, 2, 3, 4, 5 and 6 weeks. A separate group of deficient and control animals were pair-fed and killed after 3 weeks. At sacrifice, weight of the animals was recorded. Heart, kidneys, liver and gluteal muscles were excised, blotted on filter paper to remove excess blood, and the organs weighed and frozen, if not used immediately. Pyridoxal phosphate was determined in total blood, plasma and leukocytes after blood had been collected in siliconed test tubes containing a small amount of disodium versenate. Leukocytes were isolated by a variation of the method of Lie and Osgood(3) using freeze dried hemagglutinin prepared from navy beans. Tissues were homogenized in a bacterial grinder, and subjected to alkaline hydrolysis and analysed at once. Estimation of pyridoxal phosphate was performed according to the method of Boxer *et al.*(1), based on the coenzyme function of B_6-al-PO_4 in a tyrosine decarboxylase system from *Streptococcus faecalis*(4). All analyses were made in duplicate.

Results. In animals on a complete diet the concentration of pyridoxal phosphate is of comparable magnitude in heart, liver, kidney and muscle (Table I). It averages 0.82 (± 0.09) γ/g for heart, 0.86 (± 0.10) γ/g for liver, 0.93 (± 0.06) γ/g for kidney and 1.15 (± 0.21) γ/g for muscle. Pyridoxal phosphate in leukocytes averages 1.34 (± 0.13) mγ/million cells, in plasma 116.4 (± 24.1) mγ/cc and in whole blood 122.6 (± 19.2) mγ/cc.

The effect of a nutritional B_6 deficiency on body weight and the relative weight of heart, kidney and muscle is clearly evident (Tables II and III). There was a lack of gain in body weight in the deficient animals, accom-

TABLE II. Total Weight Gained and Organ Weight/100 g Body Weight in B_6 Deficient and Control Rats Fed Synthetic Complete Diet *Ad Lib.*

Group	Wk on diet	No. of animals	Wt (g) gained	% body wt		
				Heart	Liver	Kidney
Control	5–6	4	98	.28 ± .03	3.54 ± .21	.63 ± .09
Deficient	"	7	35	.34 ± .02	4.19 ± .34	.80 ± .08
Significance of diff.				<.01	<.01	<.01

TABLE III. Pyridoxal Phosphate in Organs and Blood of Vit. B_6 Deficient and Control Rats Fed Synthetic Complete Diet *Ad Lib.*

Group	Wk on diet	No. of animals	Wt (g) gain	Organ wt (g)			Concentration (γ/g)								Total content (γ)		
							Heart		Liver		Kidney		Muscle				
				Heart	Liver	Kidney	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Heart	Liver	Kidney
C*	1–2	6	33	.43	5.26	.88	.98	.90–1.10	1.27	1.00–1.80	1.20	.96–1.70	1.59	.88–2.83	.43	6.71	1.06
D	”	10	7	.38	3.87	.84	.61	.56– .80	.57	.26– .82	.44	.33– .55	.63	.32– .95	.23	2.21	.37
C	3–4	5	69	.68	7.31	1.44	1.00	.61–1.35	1.02	.75–1.73	1.00	.66–1.60	1.18	.86–1.56	.68	7.45	1.44
D	”	8	20	.53	6.75	1.24	.23	.21– .51	.26	.12– .38	.26	.15– .38	.27	.14– .37	.12	1.76	.30
C	5–6	4	98	.73	9.28	1.56	.62	.56– .70	.67	.65– .68	.63	.56– .68	.71	.67– .76	.46	6.22	.98
D	”	7	35	.68	8.20	1.56	.15	.07– .19	.14	.06– .18	.11	.01– .19	.14	.06– .18	.11	1.15	.17

Group	Wk on diet	No. of animals	Wt (g) gain	Leucocytes, mγ/mill.		Plasma, mγ/cc		Whole blood, mγ/cc	
				Mean	Range	Mean	Range	Mean	Range
C	1–2	6	33	1.39	.76–2.78	126.2	96.7–245.4	121.1	86.4–232.5
D	”	10	7	.60	.28– .85	31.8	7.5– 72.9	35.2	6.8– 72.0
C	3–4	5	69	1.42	1.17–1.56	99.3	84.0–108.9	127.9	73.0–175.2
D	”	8	20	.39	.15– .55	29.6	8.3– 42.0	22.8	7.2– 35.8
C	5–6	4	98	1.18	.87–1.42	122.4	103.5–137.5	118.2	90.0–157.5
D	”	7	35	.25	.11– .48	20.7	15.0– 24.1	16.5	~3.7– 28.8

* C = Control; D = Deficient.

TABLE IV. Pyridoxal Phosphate in Organs and Blood of Vit. B_6 Deficient and Pair Fed Control Rats Given Synthetic Complete Diet.

Group	Wk on diet	No. of animals	Wt (g) gain	Organ wt (g)			Concentration (γ/g)								Total content (γ)		
							Heart		Liver		Kidney		Muscle				
				Heart	Liver	Kidney	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Heart	Liver	Kidney
C*	3	4	76	.60	8.42	1.38	.81	.78–.88	.76	.64–.90	.76	.68–.90	1.12	1.04–1.20	.49	6.40	1.05
D	3	4	31	.63	10.06	1.62	.19	.14–.25	.10	.08–.12	.10	.08–.12	.29	.22– .48	.12	1.01	.16

Group	Wk on diet	No. of animals	Wt (g) gain	Leucocytes, mγ/mill.		Plasma, mγ/cc		Whole blood, mγ/cc	
				Mean	Range	Mean	Range	Mean	Range
C	3	4	76	1.25	.92–1.54	81.2	72.0–97.5	75.3	61.0–95.0
D	3	4	31	.21	.11– .35	31.9	24.0–40.5	16.4	11.4–21.3

* C = Control; D = Deficient.

panied by a paradoxical increase in wet weight of heart, liver and kidneys(5,6). A prompt decrease in concentration of B_6-al-PO_4, occurred in all tissues and in leukocytes and blood within 1 to 2 weeks of the developing B_6 deficiency (Table III). In this interval concentration of pyridoxal phosphate decreased to about 70% in heart, to about 45% in liver and muscle and to 30% in kidney. Total vitamin content of heart decreased to about 50%, of liver and kidney to about 35%. Concentration of B_6-al-PO_4 in leukocytes dropped to 40% and in plasma and whole blood to about 30% of control values. A marked further decrease of coenzymatically active form of Vit. B_6 occurred in the following depletion period. After 5 to 6 weeks, concentration of pyridoxal phosphate in heart averaged 15%, in liver and muscle 12% and in kidney 10% of normal. Total amounts dropped to about 20% in heart, 18% in liver and 10% in kidney. B_6-al-PO_4 levels in blood, plasma and leukocytes diminished to 15% of control values. In the pair-fed group sacrificed after 3 weeks similar results were obtained (Table IV).

Discussion. Data in the literature indicate much higher levels of Vit. B_6 in various tissues of normal rats(6,7) than those found by us for pyridoxal phosphate. Liver for instance, was reported to contain an average of 7.7 γ/g, kidney 6.6 γ/g and heart 4.6 γ/g(7). These higher values are not unexpected, since the biological assay methods in these investigations measure total Vit. B_6 content, both metabolically active and inactive components.

Boxer *et al.*(1) determined the pyridoxal phosphate content of blood of various mammalian species and found it to be highest in the rat, varying from 135 to 240 mγ/cc, (average 187 mγ/cc). This is in good agreement with our findings of 73 to 232 mγ/cc, (average 122.6 mγ/cc), when one considers the fact that our rats were on a synthetic diet, while animals used by Boxer *et al.* had consumed an optimal nonsynthetic food mixture (personal communication).

A relationship between pyridoxin supplied in the diet and codecarboxylase (pyridoxal phosphate) content of rat tissue has been reported(8). Our experiments revealed a very ready response of B_6-al-PO_4 levels in organs, leukocytes and blood to restriction of dietary Vit. B_6. This response is much more marked with the metabolically active form than with total amount of Vit. B_6. Beaton and McHenry(7), for instance, found in liver a decrease of total Vit. B_6 to about 50% within 6 weeks of deficiency. In contrast, the metabolically active pyridoxal phosphate declined to about 12% during an equally long period of nutritional B_6 depletion. The relatively greatest decrease of total Vit. B_6 content(7) and of B_6-al-PO_4 occurred in the kidney. The lowest concentration and lowest total content of pyridoxal phosphate was found in this organ, averaging only about 10% of control values after 6 weeks of B_6 deficiency. After this period the concentration of B_6-al-PO_4 in all other tissues as well as of leukocytes, blood and plasma, was not greater than 15%.

Determination of pyridoxal phosphate in either plasma, whole blood or leukocytes, can serve as a sensitive guide for assessing the nutritional status of Vit. B_6. Estimation of B_6-al-PO_4 can be done in 1 cc of blood and thus repeated examinations can be performed without necessity of sacrificing the animal. For clinical purposes, it would obviously be advantageous to use blood or plasma instead of leukocytes to avoid the time-consuming separation and counting procedures. Boxer *et al.* have, however, pointed out that the level of pyridoxal phosphate in total blood of many normal human beings is less than 10 mγ/cc, the detection limit of the method for determination of B_6-al-PO_4. Attempts are under way to improve the technic so that it can be used for analysis of such small quantities of the vitamin.

Summary. Pyridoxal phosphate levels were determined in the heart, liver, kidney, muscle, circulating leukocytes, plasma and whole blood of rats on a synthetic diet with and without added Vit. B_6. Concentration of B_6-al-PO_4 decreased in all tissues and in leukocytes, blood and plasma significantly within 1 to 2 weeks in animals on the deficient diet. The levels of pyridoxal phosphate diminished within 5 to 6 weeks to 10 to 15% of control values in all organs, circulating leukocytes, plasma and blood. It is concluded that measurement

of pyridoxal phosphate in organs as well as in leukocytes, plasma and whole blood can serve as a sensitive indicator for assessment of the nutritional state of the animals in regard to Vit. B_6.

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Hematologic Manifestations of Lysine Deficiency in Swine.* (23919)

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Lysine deficiency has been produced experimentally in rats and swine by feeding diets deficient in lysine(1), by feeding diets containing "deaminized casein" as a source of protein(2,3), and by feeding diets low in lysine to which hexahomoserine (6-hydroxy-DL-norleucine), a lysine antagonist, was added (4-7). Under all of these conditions anemia was observed. In rats Smith and Stohlman (3) found a macrocytic megaloblastic type of anemia, whereas Gillespie, Neuberger and Webster(1) reported that the anemia was hypochromic. The morphologic characteristics of the anemia in swine have not been recorded(6).

The purpose of this report is to describe the hematologic manifestations of lysine deficiency in swine.

Methods. Twenty-three swine of the Chester-White breed were housed in individual cages and were started on the experimental diet at 3 weeks of age. The diet consisted of yellow corn, 71.1%; cottonseed meal, 25%; salt mixture 3.8%; terramycin feed supplement (Bi-Con TM 10), 0.1%; and a B vitamin mixture, 0.02%. The diet was fed in an amount of 35 g/kg of body weight/day. In addition, all animals received 10 mg of choline chloride/kg of body weight daily, and 3000 units of Vit. A, 600 units of Vit. D, 1 mg of Vit. E, and 1 mg of Vit. K/kg of body weight by mouth each week. The salt mixture consisted of calcium carbonate, 1.5 g; calcium phosphate, 1.4 g; sodium chloride, 0.5 g; iron citrate, 0.3 g; manganese sulfate, 0.04 g; zinc chloride, 0.03 g; copper carbonate, 0.005 g; and cobalt carbonate, 0.0006 g. The B vitamin mixture consisted of the following (mg): thiamine hydrochloride, 0.25; riboflavin, 0.12; nicotinic acid, 1.20; pyridoxine hydrochloride, 0.20; calcium pantothenate, 0.50; inositol, 0.20; para-aminobenzoic acid, 0.10; biotin, 0.10; pteroylglutamic acid, 0.10; and cobalamine, 0.01. Five pigs were given hexahomoserine (6-hydroxy-DL-norleucine) after they had been taking the experimental diet for 112 days. The hexahomoserine was added to the diet in an amount of 0.5% of the diet. The control animals were fed a casein, sucrose, and lard diet which has been described previously(8). Plasma iron was determined by the method of Hamilton, *et al.*(9). Plasma copper was determined by the method of Gubler, *et al.*(10). Serum protein estimations were performed by a modification(11) of the method described by Weichselbaum(12).

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† Postdoctoral research fellow, Nat. Cancer Inst.

TABLE I. Summary of Observations.

Determination	Control group	Corn diet	Corn diet + hexahomoserine
No. animals	18	18	5
Red cell count, $\times 10^6$ per mm^3	7.96 ± .74 (6.5– 9.1)	6.31 ± .74 (4.4– 7.2)	3.90 (2.8– 4.7)
Hemoglobin, g %	14.6 ± .80 (13.6– 16.9)	10.5 ± 1.42 (7.4– 12.2)	6.8 (4.9– 8.5)
Vol packed red cells, ml/100 ml	45 ± 2.7 (40 – 50)	31 ± 3.2 (23 – 36)	21 (15 – 27)
Mean corpuscular vol, μ^3	56 ± 4.1 (47 – 63)	50 ± 3.5 (46 – 57)	54 (52 – 58)
Mean corpuscular hemoglobin, $\mu\mu$g	18 ± 1.9 (15 – 21)	17 ± 1.5 (14 – 20)	17 (17 – 18)
Mean corpusc. hemoglobin conc., %	33 ± 1.6 (30 – 35)	33 ± 1.5 (31 – 35)	32 (31 – 33)
Reticulocytes, %	1.1 ± .72 (0 – 6)	2.8 ± 1.58 (1 – 8)	.9 (0 – 2)
Leukocyte count, $\times 10^3$/mm^3	13.8 ± 6.18 (11 – 26)	15.3 ± 7.17 (8 – 33)	9.3 (6 – 12)
Plasma iron, μg %	169 ±38.8 (102 –480)	160 ±34.5 (116 –212)	156 (61 –290)
Plasma copper, μg %	206 ±26.3 (113 –288)	148 ±16.2 (126 –177)	157 (84 –185)
Total serum protein, g %	5.49 ± .17 (5.3– 6.0)	4.74 ± .31 (4.2– 5.3)	3.82 (3.3– 4.2)
Serum albumin, g %	2.92 ± .30 (2.4– 3.2)	1.63 ± .42 (1.1– 2.1)	1.40 (1.3– 1.5)

Values refer to mean ± 1 stand. dev. Figures in parentheses refer to range.

Hemoglobin was determined as cyanmethemoglobin by the use of a photometer.

Results. The hematologic observations are summarized in Table I. All of the animals were anemic after 4 weeks on the corn diet. The anemia was mild in degree and constant in severity with little tendency to progress with time (Fig. 1 and 2). The morphology of the erythrocytes was within normal limits, as were the reticulocyte, leukocyte and plasma iron values. A moderate but significant degree of hypocupremia ($t = 4.7$; $P < .001$) and hypoproteinemia ($t = 6.9$; $P < .001$) were present. The hypoproteinemia was due to a reduction in serum albumin.

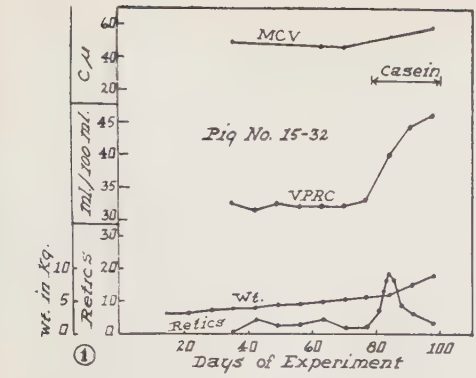
Bone marrow differential counts on smears obtained from aspirated material were within normal limits. Histologic sections of sternal, rib, vertebral and femoral bone marrow, liver, lymph node, spleen, heart and skeletal muscle were prepared from 3 untreated pigs and from one pig treated with lysine. Examination of the bone marrow sections revealed normal cellularity. No significant histologic abnormalities were noted in the other tissues examined.

Six of the anemic pigs were treated with casein by substituting purified casein for the 25% cottonseed meal in the diet. In all 6 of the pigs this dietary alteration was followed by reticulocytosis (6 to 18%) and alleviation

TABLE II. Hematologic Response to Administration of Casein and Lysine.

	Before therapy			After therapy			
				Retic. max		V.P.R.C.	
Pig. No.	V.P.R.C., ml/100 ml	Retic., %	Therapy	Day	%	Day	ml/100 ml
15–17	30	2	Casein	6	6	45	45
19	24	3	"	4	10	46	46
32	33	2	"	5	18	20	46
33	35	2	"	4	18	12	44
34	33	1	"	5	16	12	43
35	34	2	"	6	18	12	43
36	31	2	Lysine	3	12	14	39
37	34	2	"	4	12	11	39

V.P.R.C. refers to vol of packed red cells. Retic. max refers to reticulocyte maximum.



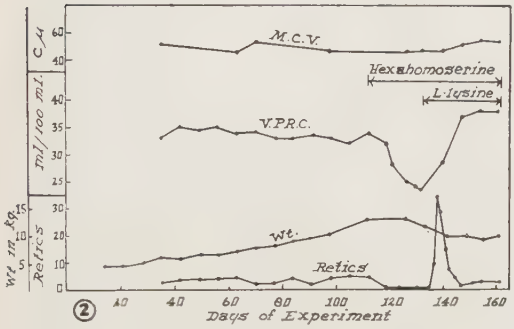


FIG. 1. Hematologic response to substitution of casein for cottonseed meal.

FIG. 2. Development of severe anemia following administration of hexahomoserine and response to administration of L-lysine.

M.C.V., mean corpuscular vol; V.P.R.C., vol of packed red cells; Wt, body wt; retics, reticulocytes.

of the anemia (Table II). The hematologic response of one such pig is shown in detail in Fig. 1.

Two pigs were treated with lysine. Two g of L-lysine were added to each 100 g of diet. In both instances this was followed by reticulocytosis and an increase in the volume of packed red cells (V.P.R.C.) (Table II). However, in neither of the 2 pigs did the V.P.R.C. reach levels as high as following substitution of the cottonseed meal with casein.

Five pigs were given hexahomoserine after they had been taking the corn diet for 112 days. In all 5 pigs a severe normocytic, normochromic anemia and a mild to moderate degree of leukopenia developed (Table I). The plasma iron levels remained within normal limits.

Histologic sections of the tissues were prepared from 4 of the severely anemic animals given hexahomoserine. The bone marrow revealed normal cellularity. No significant abnormalities were noted in the other tissues examined. Bone marrow differential counts on thin smears obtained from aspirated material were within normal limits.

One of the pigs given hexahomoserine was treated with lysine. Two g of L-lysine were added to each 100 g of diet and the hexahomoserine was continued. Administration of lysine was followed by reticulocytosis (maximum, 35%) and complete alleviation of the anemia (Fig. 2).

Discussion. It is evident from the data presented that lysine is essential for normal erythropoiesis in swine. The animals fed the corn diet developed anemia and, when hexahomoserine was added, the volume of packed red cells decreased rapidly. Addition of lysine to the diet, either in the presence or absence of hexahomoserine, was followed by reticulocytosis and rapid alleviation of the anemia.

The anemia observed was normocytic and normochromic and was unaccompanied by reticulocytosis and is thus similar morphologically to that observed in protein-deficient (13), tryptophane-deficient(14), and in niacin-deficient(15) swine.

Chronic protein-deficiency in pigs is accompanied by a moderate degree of hypocupremia and hypoferremia(13). Both of these elements are bound to specific proteins under normal conditions. Copper is present in plasma as a copper protein, ceruloplasmin. Iron is bound to the iron-transport protein, transferrin. It is reasonable to suppose that, in protein deficiency, hypocupremia and hypoferremia are the consequence of impaired synthesis of the specific binding proteins. In the animals deficient in lysine the degree of hypocupremia was comparable to that observed in protein-deficiency. From this it would appear that lysine is essential for ceruloplasmin synthesis. The fact that hypoferremia was not present in the lysine-deficient animals suggests that lysine is not of critical importance in synthesis of transferrin.

The earlier studies on the morphology of red cells in lysine deficient rats were limited to an estimation of the color index. Gillespie *et al.*(1) reported that the color index was

decreased; Smith and Stohlman(3) found the index to be increased. This discrepancy may have been due to inaccuracies in hemoglobinometry. However, the latter workers also observed a great variety of cellular abnormalities in the blood, such as anisocytosis, poikilocytosis, polychromatophilia, Howell-Jolly bodies, nucleated red cells, myeloblasts and myelocytes. Such abnormalities were not observed in the blood of the anemic swine. In the rats the anemia was accompanied by a reticulocytosis of 5 to 25%. The explanation for these differences between the 2 species is not obvious.

Summary. 1. Twenty-three swine were fed a diet deficient in lysine. In addition, 5 swine were given hexahomoserine (6-hydroxy-DL-norleucine), a lysine antagonist. 2. All animals developed a normocytic, normochromic anemia, which was accompanied by hypoalbuminemia and hypocupremia. No alterations from the normal were noted in the plasma iron concentration or in bone marrow morphology. The anemia responded rapidly to administration of lysine. 3. The data presented indicate that lysine is essential for normal erythropoiesis in swine.

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